IMMUNOPOTENTIATION STUDIES WITH *VIBRIO CHOLERAE*

CAROL E. LAUCHLAN

Presented for the degree of Doctor of Philosophy
in the Faculty of Science, University of Glasgow.

Department of Microbiology

September 1978

PREFACE

This thesis is the original work of the author. She wishes to acknowledge that all animal experimentation was done in collaboration with Dr. D.E.S. Stewart-Tull.

Carol E. Lauchlan

CONTENTS

ACKNOWLEDGEMENTS

My thanks are firstly extended to Dr. D.E.S. Stewart-Tull, who supervised this project with enthusiasm and interest, despite having a multitude of other commitments. His good humour after seemingly endless hours in the operating theatre was very much appreciated.

I am also grateful to Professor A.C. Wardlaw, both for reading this manuscript, and for his help in solving many problems, academic and otherwise, during my stay in his department.

This research was supported by a studentship from the Medical Research Council, to whom I express my thanks.

I owe particular thanks to Mrs. A. Strachan for typing this thesis so quickly and accurately, and to Mr. I. McKie for his excellent photographic work.

Thanks are also due to many other members of the Microbiology Department, both for their friendship and for their willing offers of help. This is particularly true of the animal house staff, who regularly came to my assistance when it was least convenient for them.

Finally I wish to express my sincere thanks to my parents for all their encouragement and financial help.

OBJECT OF RESEARCH

The main shortcomings in the present cholera immunisation schemes are the incomplete protection afforded by currently available vaccines and the short duration of the immunity stimulated.

This study (in laboratory animals) was undertaken to investigate possible alternatives to the current commercial vaccine which is a heat-killed, saline suspension of V. cholerae.

The following were considered:-

1. Use of cholera toxoid as immunogen, either in preference to or in conjunction with heat-killed V. cholerae cells. Cholera toxin is known to be responsible for the pathogenesis of cholera and therefore toxoid-containing vaccines might provide an alternative to the whole cell vaccine.
2. Use of oral or combined i/m and oral immunisation schedules. Cholera vibrios and toxin are completely confined to the lumen of the intestine during infection[2]; therefore it is important to stimulate a gut-associated antibody response. Oral immunisation may stimulate such a response, perhaps better than the s/c or i/m routes of immunisation currently employed.
3. Incorporation of vaccines into adjuvant mixtures. Protection against cholera has been associated with high serum antibody titres so that stimulation of the immune response to the protein enterotoxin by adjuvants was thought to be a worthwhile line of investigation.

Wherever possible the practicality of using a vaccine in the field was considered. The main aim was to obtain an immunisation schedule which could be put into operation with minimal involvement of qualified personnel. For this reason the oral route of administration was strongly favoured.

INTRODUCTION

Page

MATERIALS AND METHODS

Page

INDEX OF TABLES

INDEX OF FIGURES

Page

INDEX OF PLATES

LIST OF ABBREVIATIONS

"AB"	Ogawa serotype
"AB(c)"	Ogawa-Hikojima serotype
"AC"	Inaba serotype
ATP	adenosine triphosphate
A.U.	antitoxin unit
B.D.	blueing dose
B.G.S.	borate gelatin saline
cAMP	cyclic adenosine monophosphate
C.F.T.	complement fixation test
C.V.	coefficient of variation
DANSyl	dimethylaminonaphthalene 5-sulphonyl
E	extinction
ED_{50}	50% effective dose
GDP	guanosine diphosphate
Gm_1	galactosyl-N-acetylgalactosaminyl- N-acetylneuraminyl galactosyglucosyl ceramide
Gm_3	N-acetylneuramonyl galactosyl glucosyl ceramide
GTP	guanosine triphosphate
G.P.	guinea-pig
"H"	flagellar antigen
H.U.	haemolytic unit
i/d	intradermal
Ig	immunoglobulin
i/m	intramuscular
i/p	intraperitoneal
I.U.	international unit
i/v	intravenous

KDO	ketodeoxyoctonate
L_b	limit of blueing
LPS	lipopolysaccharide
2ME	2-mercaptoethanol
NAD	nicotinamide adenine dinucleotide
NCTC	National Collection of Type Cultures
N.I.H.	National Institute of Health
"O"	somatic antigen
per os	by mouth
P.F.	permeability factor
PGE_1	prostaglandin E_1
PHA	passive haemagglutination
R	rough
ρ(rho)	rugose
RF	ribosome containing fraction
S	smooth
s/c	subcutaneous
S.E.M.	standard error of the mean
TCBS	thiosulphate-citrate-bile salt sucrose
Tris	Tris-(hydroxymethyl)-methylamine
V.B.S.	veronal buffered saline
W.H.O.	World Health Organisation

INTRODUCTION

I. GENERAL INTRODUCTION

The existence of cholera, or a similar disease, in the ancient civilisations of India and possibly China was suggested by Pollitzer (1959), in an historical review. Cholera was a divine judgement on the decrease in "the strength of virtues and merits on earth" - according to one piece of evidence dating from the 9th century A.D. From the 16th century onward the epidemic nature of cholera was suggested by reports from the west coast of India. With the establishment of a hospital board in Madras and Calcutta in 1786, the opportunity arose to monitor the disease and its spread more carefully.

By 1819 the Bengal Medical Board accepted that cholera was caused by a "pestilential virus", but did not agree with its being a contagious disease. Neale (1831) applied the contagion theory to cholera and Boehm (1838) was first to claim to have seen the causative organism. This claim was subsequently made by other workers including both Pacini and Hassal (1854), Leyden (1866), and finally Klob (1867) described intestinal organisms identical to Vibrio cholerae. Through the studies of Koch (1884) on cholera victims in Egypt and India, it became generally accepted that V. cholerae was the causative agent. He reported that comma-shaped organisms increased in numbers in the stool during infection and gradually disappeared on recovery. The bacteria appeared to be almost invariably present in the intestines of the cholera victim and absent in any other morbid disease (cited from Pollitzer, 1959).

Until the end of the 18th century cholera, as far as is known, was contained largely within India and neighbouring regions. From this time, however, there have been a succession of cholera pandemics,

the first six of which were described in detail by Pollitzer (1959). In this author's account the approximate eras of these pandemics were as follows:

1st	Pandemic	: 1817-1824
2nd	"	: 1829-1850
3rd	"	: 1852-1860
4th	"	: 1863-1879
5th	"	: 1881-1896
6th	"	: 1899-1923

In each case the origin of the pandemic could be traced to the west coast of India, whence, following a sudden increase in the rate of cholera infection, the disease spread throughout India and beyond. With few exceptions almost every country in the world has been affected by cholera at some time during the course of these pandemics. Often the spread of the disease was associated with trade routes or movement of troops. It was characterised always by high infection and mortality rates in each infected community. For example, cholera caused 9,000 deaths, mostly within a few days, in Grand Canary Island in May 1851, and claimed 1,700 victims from Bathurst in Gambia in 1869, out of a population of 5,000.

The seventh pandemic of cholera began in 1961, after an absence from most of the world since the nineteen-twenties. During the intervening years cholera was confined mainly to the deltas of the Ganges and Brahmaputra rivers, and even there only occasionally reached epidemic proportions. Since 1961, the disease has gradually spread over a large area of the world; a process which can be divided into several distinct stages:-

1st Period: 1961-1962:- during which the disease was recognised in all the countries of south east Asia, e.g. the Phillipines.

2nd Period: 1963-1969:- the disease reached the countries of mainland Asia, e.g. Thailand.

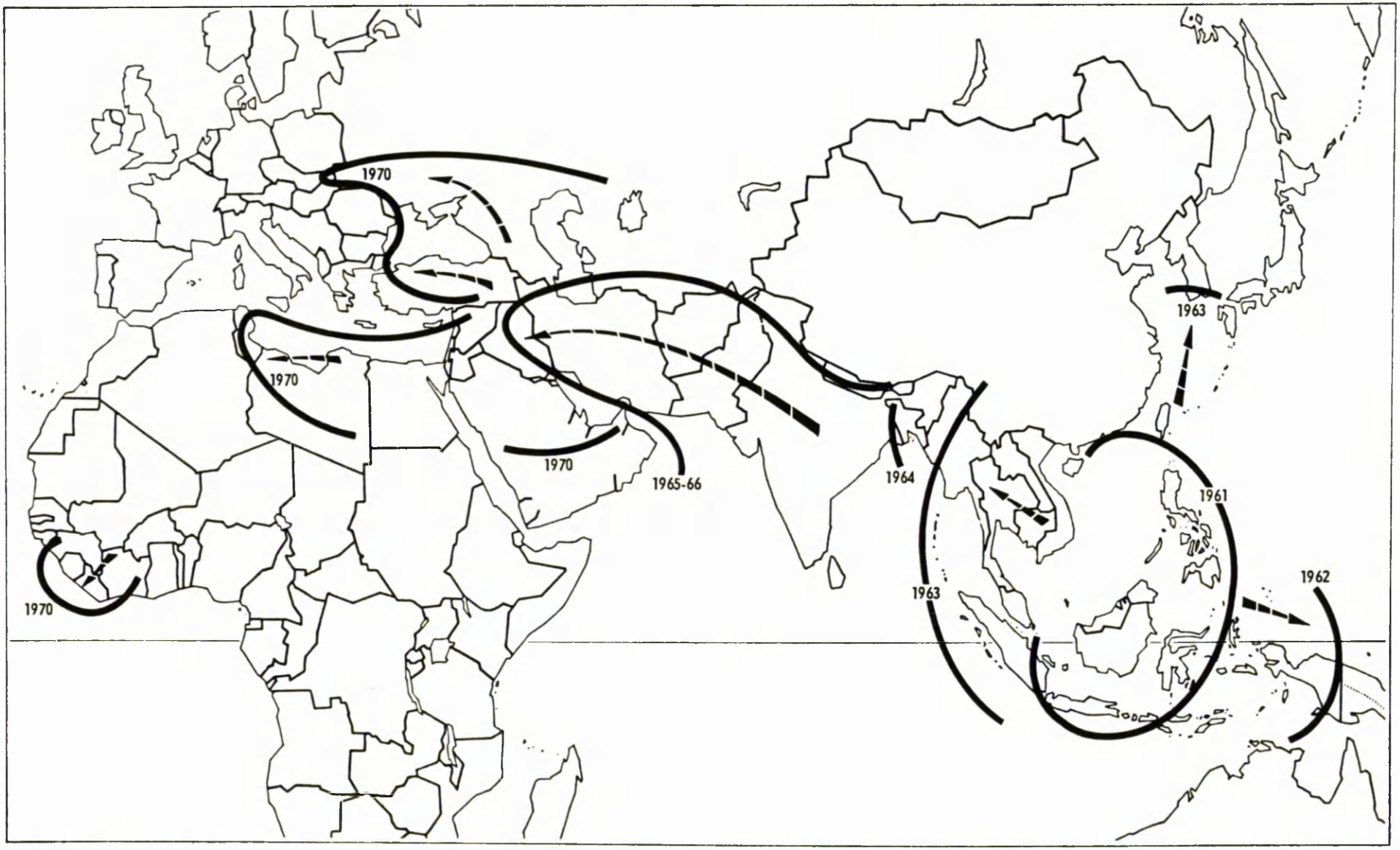
3rd Period: 1970-197-:- there was a significant rise in the incidence of cholera in the Middle-East, Africa and Europe (Plate 1).

The classical biotype of V. cholerae, identified by Koch in 1884, was characterised by its lack of haemolytic activity normally associated with the apathogenic vibrio strains, commonly found in nature. In 1906, however, Gotschlich isolated a haemolytic vibrio strain from cholera victims in Egypt, and eventually Moor (1939) found endemic cholera in Indonesia was due to the haemolytic (or eltor) biotype of V. cholerae. Recently, this eltor biotype of V. cholerae has lost its haemolytic characteristic and is now distinguished from the classical V. cholerae by its (a) resistance to Mukerjee's phage IV (Mukerjee, 1963), (b) resistance to polymyxin (Gan and Tjia, 1963) and (c) ability to agglutinate chicken red blood cells (Finkelstein and Mukerjee, 1963).

It soon became apparent that the eltor variety of the cholera vibrio was more prevalent, in the seventh pandemic, than classical V. cholerae. It is considered to be the major etiological agent of cholera in the present pandemic. The eltor biotype differs from classical V. cholerae in having a longer survival time in the environment and in being more resistant to antibiotics. It also causes a higher incidence of asymptomatic infection than the classical V. cholerae; infection:case rates of 4:1 for classical Inaba infection, and 36:1 for eltor Ogawa infection were found by Bart et al (1970). Consequently, there are more undetected and untreated cholera cases which preserve a reservoir of infection in eltor epidemics.

Plate 1. World map showing the spread of cholera during the seventh pandemic.

EXTENSION OF CHOLERA 1961-1970



The W.H.O. estimates that half of the world's population lives in or near the cholera endemic areas of Asia, so the disease obviously poses an immense public health problem. Because of the enormity of the problem, means of prevention and treatment of cholera have been urgently sought. Treatment of the disease is now so refined that infection with V. cholerae need not be fatal.

Unfortunately, cholera is endemic where high population density and poor sanitation encourage infection, and where restricted medical facilities hinder large-scale treatment. While these conditions prevail, there is little hope of eradication of the disease. Emphasis should therefore be placed on the production of an effective vaccine which is capable of eliciting long-term protection against the disease.

II. TYPE OF INFECTION

1. The Causative Organism

Cholera is caused by the Gram-negative, uniflagellate, facultatively anaerobic rods belonging to the family Vibrionaceae (Véron, 1965; Bergey, 1974). It is contained in the genus Vibrio (Pacini, 1854). The species Vibrio cholerae is subdivided into four biotypes:- (a) V. cholerae biotype cholerae, (b) V. cholerae biotype eltor (Pribram, 1919), (c) V. cholerae biotype proteus (Buchner, 1885), (d) V. cholerae biotype albensis (Lehmann and Neumann, 1896).

Only biotypes (a) and (b) are associated with human cholera. Biotype (c) causes gastroenteritis in humans and biotype (d) is pathogenic only to certain animals, e.g. guinea-pigs and pigeons. All four biotypes are intestinal pathogens.

On primary isolation from patients, vibrio colonies are smooth, glistening and translucent, and by oblique light show a typical

greenish to red-bronze iridescence and fine granular translucency. Colonial variants are either opaque and corrugated (rugose variants) or rough. The rugose variant is seen especially in old cultures.

The majority of vibrio strains may be classified into six antigenic "O" groups. The cholera vibrios (biotypes cholerae and eltor) belong to the "O" : I group (see Figure 1); strains belonging to other groups are usually called "non-cholera vibrios."

A. Antigenic Constitution

V. cholerae possesses both "H" and "O" antigens. The "H" antigens are non-specific, and are shared with non-cholera vibrios. The diagnosis of cholera is dependent, therefore, on the use of "O"-specific antisera. Lyles and Gardner (1958) found that the smooth (S) forms of V. cholerae were agglutinable with "O" antisera, while the rough (R) and rugose (ρ) forms were not agglutinated unless preheated at 100°C for one hour. It was suggested that preheating inactivated a thermolabile inhibitor of agglutination present on the surface of R and ρ forms.

Gallut (1962) implicated the cell-wall as the site of the specific "O" antigen in both the classical and eltor organisms. Spontaneous removal of part of the cell-wall on suspension of the vibrios in isotonic saline + glucose solution, and their incubation at 20°C or 37°C, resulted in a considerable reduction of "O" agglutination; the specific agglutinogen was recoverable from the autolysates. Pant (1968) confirmed this localisation of the specific antigens by removing the outer membrane of V. cholerae with a 0.005 percent sodium desoxycholate solution. After inoculation of rabbits with the extract it was possible to recover "O" specific antisera from these rabbits in high titre.

B. Variation in Serotype

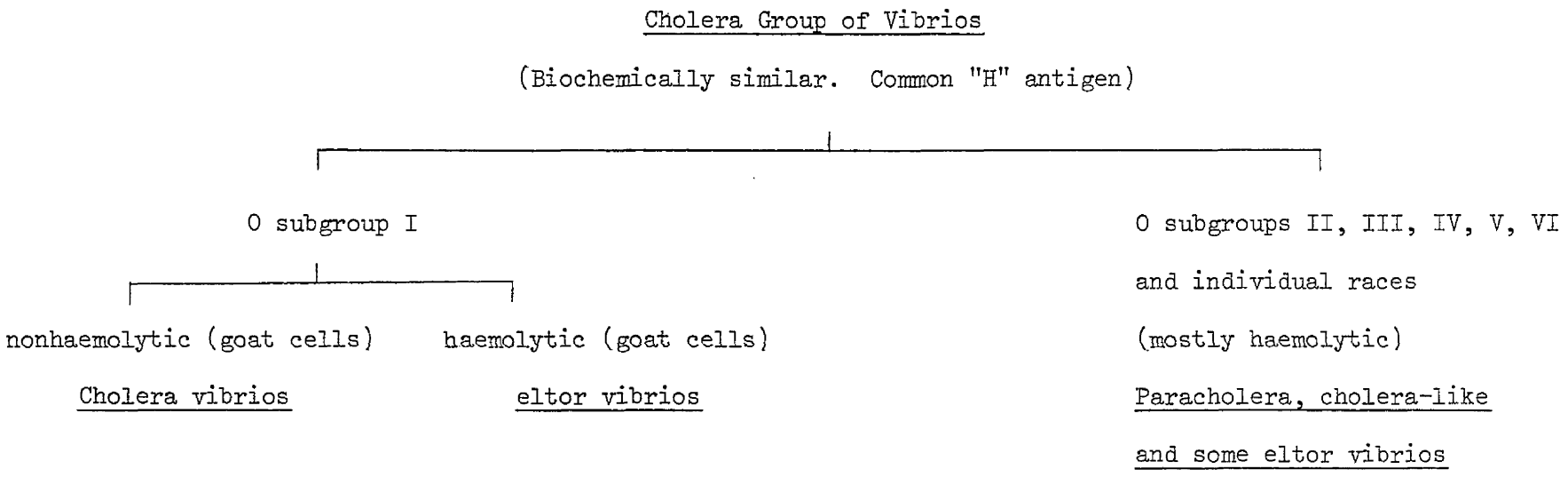
Kauffmann (1950) divided the cholera vibrios of Gardner and Venkatraman's "O" group I (Gardner and Venkatraman, 1935) (Fig 1) into two "O" antigenic forms, Inaba and Ogawa, by agglutination and agglutinin absorption tests. The Inaba serotype was given the antigenic formula "AC", and the Ogawa (or Ogawa-Hikojima) serotype the formula "AB(C)". Formerly, Ogawa strains were designated as "AB" and Hikojima as "ABC", however, Kauffmann (1950) observed that Ogawa organisms grown at 20°C could totally absorb the antibody in Inaba sera. The variability between Ogawa and Hikojima organisms seemed to be largely phenotypic, with a quantitative antigenic difference.

However serological interconversions were reported for both Ogawa and Inaba serotypes by Sack and Miller (1969) using germ-free mice. It was noted that Ogawa organisms were recovered from mice infected with the Inaba serotype and vice versa. These serological conversions appeared to be correlated with the appearance of agglutinating antibody in the serum and were prevented, or significantly retarded, when antibody formation was suppressed with cyclophosphamide, and ~~was~~ were accelerated by prior vaccination with purified antigen homologous to the infecting strain. Rough to smooth reversions were found also, with both serotypes (Sack and Miller, 1969; Miller et al, 1972).

C. Chemical Nature of the Somatic Antigens

The exact chemical structures of the "O"-somatic antigens of V. cholerae are still unknown, despite the efforts of numerous research workers. Watanabe and Verwey (1965) isolated a lipopolysaccharide (LPS) antigen, from the culture supernate of an Ogawa serotype of the eltor vibrio; this LPS appeared to be biochemically and immunologically

Figure 1. Division of cholera vibrios in terms of "O" antigenicity (Gardner and Venkatraman, 1935)



homogeneous. The LPS antigen, isolated by sequential ammonium sulphate precipitation, deproteinisation by phenol extraction, lipid removal by chloroform-methanol, and cold ethanol fractionation, contained approximately 60% carbohydrate (as glucose), which was composed of glucose, glucosamine and glucuronic acid. The nitrogen content was low and the principal amino acid was glutamic acid.

Verwey et al (1965) similarly tried to obtain an antigenic extract from the Inaba serotype of an eltor vibrio. They extracted such a substance from whole Inaba cells by treatment with urea, and sonication before precipitation with ammonium sulphate. In comparison to the Ogawa LPS antigen obtained from culture supernates, the Inaba antigen, obtained by these authors, was more tightly bound to the cells, and was severely damaged by phenol extraction. The Inaba antigen also contained more nitrogen and lipid, but less carbohydrate than the Ogawa LPS antigen. It was proposed that this Inaba antigen was similar to a Boivin-antigen, consisting of a protein-carbohydrate-lipid complex, and that a heat-labile, phenol-sensitive moiety (probably protein) was necessary for full activity.

More recently, Jackson and Redmond (1971) made immunochemical studies on Inaba LPS. Heptose, glucose, glucosamine, mannose and glycerol were shown to be the major components, and lipid A accounted for 30% of the preparation. However, a further 30% of the weight of this LPS was not identified and ketodeoxyoctonate (KDO) and galactose were not detected. It was therefore suggested that there might be fundamental differences between the structure of the core region of vibrio LPS and that of Salmonella LPS (Westphal, 1975).

Sur, Maiti and Chatterjee (1974) reported chemical analysis of isolated V. cholerae cell envelopes as follows: protein, 42-43%;

total carbohydrate, 9.2%; hexose, 5.2%; hexosamine, 3.5%; lipid, 18%; phosphorous, 1.4%; calcium, 0.18%; magnesium, 0.12%. As before, the total envelope weight could not be accounted for. This anomaly may be explained by the discovery by Redmond (1975) of a highly-labile sugar, 4-amino-4,6-dideoxy-D-mannose, amounting to at least 6%, in the LPS of V. cholerae 569B Inaba.

Raziudin and Kawasak (1976) presented detailed analyses of V. cholerae cell envelope LPS. The absence of KDO, the typical linking sugar between the polysaccharide and lipid moieties in enterobacterial LPS, and galactose, a typical core sugar in other gram-negative bacteria, was again outstanding.

It is unfortunate that a comprehensive analysis of the V. cholerae cell envelope has not been made. Characterisation of the protective somatic antigen(s), believed to be located on the envelope, is potentially of great importance to the development of an effective cholera vaccine.

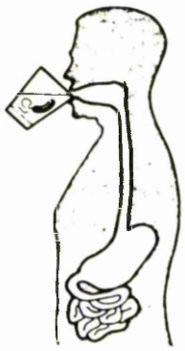
2. The Disease

A. Colonisation of the Intestine and Toxin Production

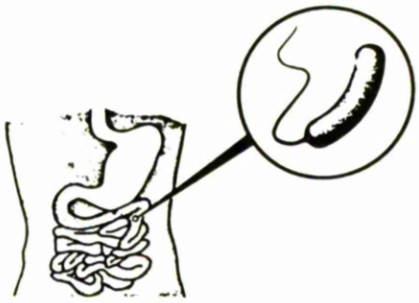
Cholera infection is via the orogastric route, the disease being dependent on the ingestion of live vibrios in water or food, and their subsequent multiplication in and colonisation of the small intestine (Carpenter, 1970; Carpenter, Greenough and Gordon, 1974; Nalin, 1975) (Plate 2);V. cholerae organisms are very acid-sensitive so that protection from gastric acidity, e.g. within a bolus of food, is required for their safe passage into the small intestine. The organism grows well at the slightly alkaline pH (7-8) of the small bowel, and is resistant to high bile-salt concentrations, so that rapid multiplication

Plate 2. Schematic diagram of the pathophysiology of cholera. (Nalin, 1975).

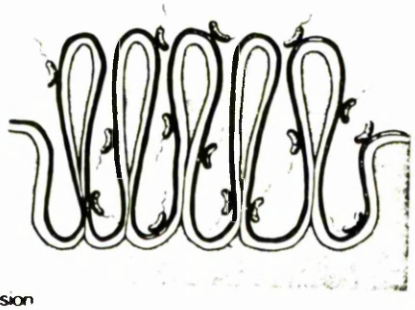
Cholera Pathophysiology



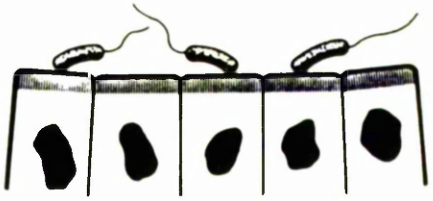
Infection



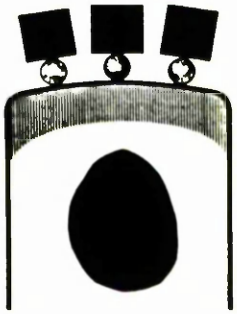
Colonization



Adhesion

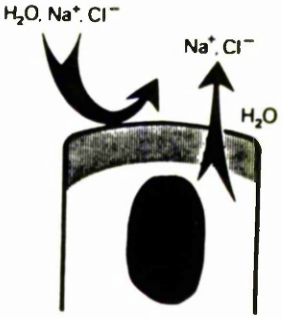


Elaboration



Binding

Activation



Flux change

may occur. Growth of the cholera vibrio in the small intestine is accompanied by the production of an exo-enterotoxin which is the agent responsible for the disease symptoms.

During cholera infection V. cholerae remains localised within the intestine, and although no penetration of the tissues occurs, Freter (1969) demonstrated that 50% of the infecting vibrios in rabbit ileal loops adhered strongly to the intestinal lining. Prior mixing of vibrios with specific antiserum while not affecting their viability, prevented their adhesion to the mucosal surface.

The mediator of vibrio adhesion has variously been suggested as:

(a) Mucinase production. The inner surface of the intestine is lined with a mucus layer. The production of mucinase may enable the vibrio to penetrate this layer and avoid removal by peristalsis. However production of this enzyme is not restricted to pathogenic vibrios (Lankford, 1960).

(b) Neuraminidase production. Durikhin, Popova and Kobrinskii (1976) found a high degree of correlation between synthesis of both enterotoxin and neuraminidase. It was suggested that these products acted cooperatively during the development of cholera, since neuraminidase destroyed the protective mucin in the small intestine and increased the content of available enterotoxin receptor sites in the epithelial cell membranes. This property of the enzyme was rejected, however, by Holmgren et al (1975,a) (see p. 40).

(c) Pili. Pili are known to be responsible for the adhesion of several enteric organisms to the intestinal epithelium (Duguid and Gillies, 1957). However, not all pathogenic vibrio strains possess these structures (Finkelstein, 1973).

(d) Flagella. A series of recent reports has suggested a correlation between possession of flagella and adhesion to mucosal surfaces by vibrios. Guentzel and Berry (1975) demonstrated a relation between motility and virulence. Non-motile V. cholerae mutants had a decreased ability to kill suckling mice despite the fact that they produced enterotoxin. It was suggested that the non-motility of these strains greatly decreased their ability to adsorb to the mouse intestinal epithelium.

Jones and Freter (1976) and Freter and Jones (1976) found that non-flagellate, mutant strains of V. cholerae lacked the ability to adhere to the surface of epithelial cells. They also found that adhesion of motile V. cholerae strains was significantly reduced by L-fucose and to a lesser degree by D-mannose. They concluded that vibrios possessed receptors on their flagella which bound to L-fucose residues on epithelial cell membranes. Flagella may also be important for propelling the vibrios toward these binding sites, since studies by Jones, Abrams and Freter (1976) demonstrated that vibrio attachment resulted from chance collision with the brush border.

The results of Nelson, Clements and Finkelstein (1976), however, contradict the observations of Jones and Freter (1976). Using electron microscopy Nelson's group demonstrated vibrios bound to the epithelial surface in a horizontal position with their flagella extending into the lumen of the intestine. It was suggested that since the cholera vibrio was propelled by a single polar flagellum, its first contact with the intestinal surface would be via the "front-end". After the initial binding, the vibrio was envisaged as aligning parallel to the surface to allow greater contact. During this sequence of events it is

difficult to imagine how a binding site on the flagellum, which apparently was not in contact with the epithelial cells, could be effective, as proposed by Jones and Freter (1976).

(e) Chemotaxis. Allweiss et al (1977) suggested that chemotaxis might play a role in the initial attraction of V. cholerae to the intestinal mucosa. They produced evidence of chemotaxis in V. cholerae which was absent from three mutant strains.

Whatever the factors involved in the adhesion of V. cholerae, it is probably essential to the virulence of the organism. Freter (1972) suggested that only vibrios adsorbed to the mucosal surface were involved in the pathogenesis of experimental cholera. This suggestion was confirmed by Schrank and Verwey (1976) who showed that the villi and the intervillous spaces of the gut were usually lined with a layer of mucoid material which isolated the epithelial cells from the intestinal contents. Onset of fluid accumulation in the gut of rabbits infected with V. cholerae, was concomitant with the establishment of large accumulations of organisms in the intervillous spaces and crypts after the successful penetration of the mucous layer. However, in rabbits immunised with a cell-wall antigen of V. cholerae, or injected with anti-cell-wall serum, there was clumping of the vibrios in the lumen and restricted distribution in the lumen and luminal edge of the mucous zone. The antibodies were non-bacteriolytic in vivo and were presumably effective through their prevention of the vibrio-mucosal surface interaction. The adhesion of the vibrios to the mucosal surface results in the deposition of cholera enterotoxin close to the toxin-receptor molecules in the epithelial cell membranes. Aziz and Mosley (1972) showed that relatively small amounts of the toxin produced by infecting vibrios were recovered in the stool of cholera patients, which suggested enterotoxin-binding was highly efficient.

The localisation of the cholera enterotoxin in the body, during infection, was investigated by Vaughan-Williams and Dohadwalla (1969) in two-day-old rabbit littermates. Pairs of these animals were linked by cross-circulation (blood from one animal was fed, via a roller pump, from the heart side of the left carotid artery to the head side of the right carotid artery of its mate, and vice versa), and one animal, the donor, was given an intraintestinal inoculation either of 3×10^7 V. cholerae organisms or sterile culture medium; the partner remained uninoculated. Fluid accumulation was observed in the intestines of both inoculated and uninoculated rabbits. These workers concluded that intestinal infection of infant rabbits with V. cholerae led to the appearance of a choleragenic agent in the blood, and that intraluminal instillation of a V. cholerae cell-lysate had a similar effect.

Pierce, Greenough and Carpenter (1971), however, could find no trace of cholera enterotoxin in either thoracic duct lymph or in mesenteric blood, during experimental cholera. This indicated that the enterotoxin was localised in the intestine, and that no other tissue of the body was exposed to cholera toxin during infection.

B. Production of Clinical Disease

The events preceding the development of clinical cholera are:- (a) ingestion of viable V. cholerae, (b) colonisation of the small intestine and (c) production of enterotoxin.

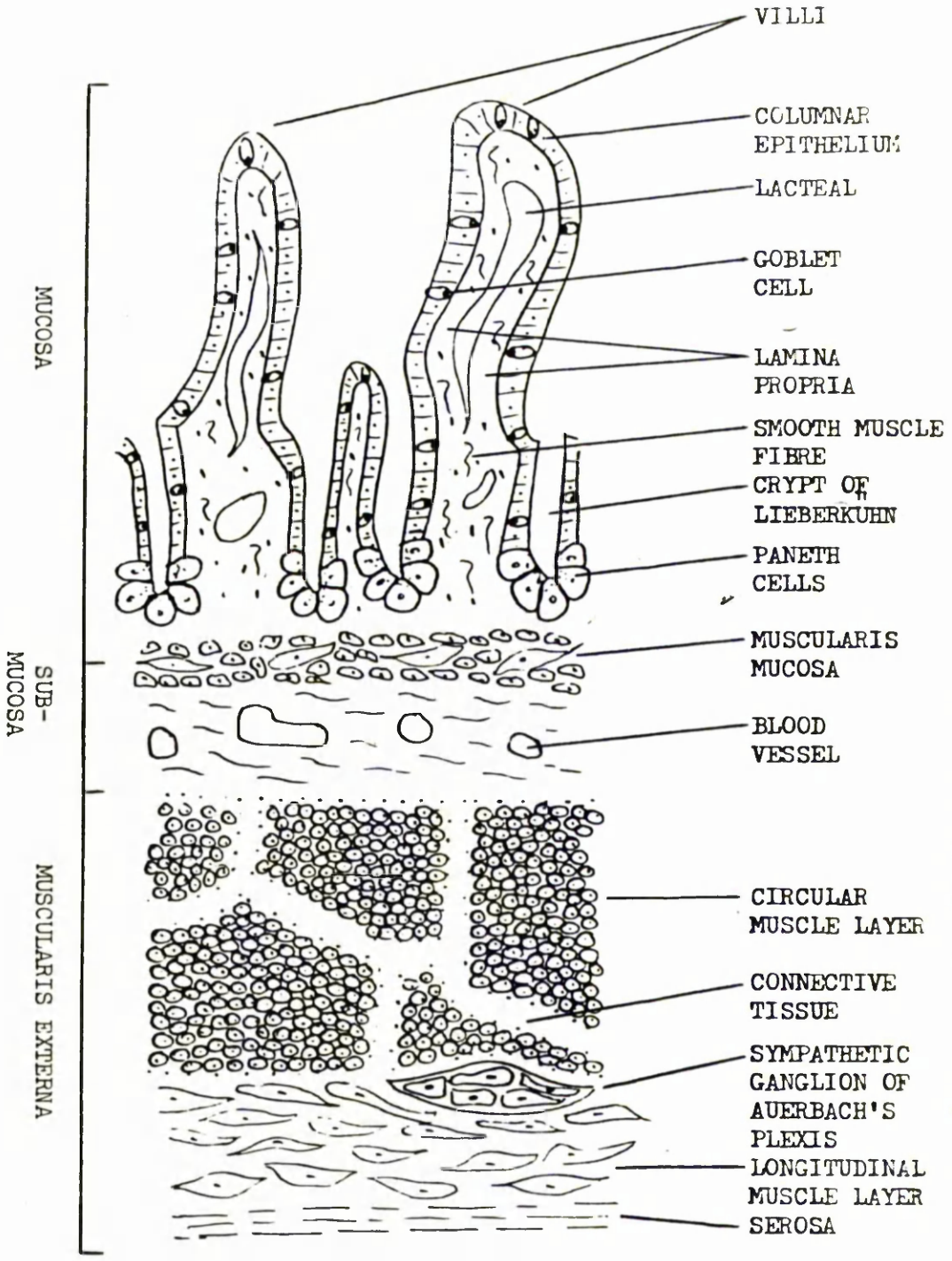
The enterotoxin acts on the mucosal cells of the small intestine causing them to secrete large quantities of isotonic fluid. The rate of fluid loss (which may be up to 1 litre per hour) ultimately exceeds the rate of reabsorption in the colon, causing a net fluid deficit in the body which is responsible for the entire range of clinical manifestations of cholera (Carpenter, 1970; Carpenter et al, 1974)

Fluid loss, according to the studies of Carpenter et al (1968) and Banwell et al (1970), occurred only in the small intestine, both in experimental canine cholera and in human cholera. Gross structural damage to the intestinal wall was absent both in man (Gangarosa et al, 1960) and experimental animals (Norris and Majno, 1968; Elliot et al, 1970) during cholera infection. Elliot et al (1970) noted several histological changes in the small intestine including the depletion of mucus in goblet cells, slight dilation of the crypts and the villous capillaries, and minimal oedema of the lamina propria. Peterson, LoSpalluto and Finkelstein (1972) studied localisation of enterotoxin in vivo, by immunofluorescence techniques. There appeared to be binding to the entire mucosal surface of the villus and crypt areas, while no penetration into the epithelium or lamina propria was observed.

Whether enterotoxin acts primarily on crypt or villus cells (Fig 2) is uncertain. De Jonge (1975) believed that both cell types were involved, while Weiser and Quill (1975) were in favour of the theory of adenylate cyclase stimulation occurring only in villus cells. Elliott et al (1970), Schwartz, Kimberg and Ware (1975) and Field (1976) however, concluded that enterotoxin acted primarily on crypt cells. The situation remains to be clarified.

Nelson et al (1976) studied the colonisation of the small intestine of infant and adult rabbits by V. cholerae, using scanning and transmission electron microscopy. They demonstrated a lag period of 1 hr before significant numbers of bacteria attached to the adult intestinal epithelium. This lag was absent in the infant rabbit where colonisation began immediately on infection, and where greater concentrations of vibrios accumulated than in the adult. It was suggested that natural differences in susceptibility of the intestinal

Figure 2. Histological transverse section of the small intestine



(Freeman and Bracegirdle, 1967)

surface to bacterial attachment could be important in the different morbidity rates of infant and adult human beings for such enteric diseases as cholera.

Nelson et al (1976) noted that after the initial attachment of vibrios there was a steady increase in their numbers, until between 4 and 7 hours post-infection when there was a maximum number of adherent bacteria several layers thick. These formed a dense mat over much of the villi. A rapid decline in the number of bound V. cholerae was subsequently observed and by 12 to 16 hr post-infection only a few bacteria were seen on the surface of the villi. After removal of the vibrios the adult rabbit villus appeared rough and patchy in comparison to control preparations, and the infant rabbit villus had a "fuzzy" appearance due to elongated microvilli.

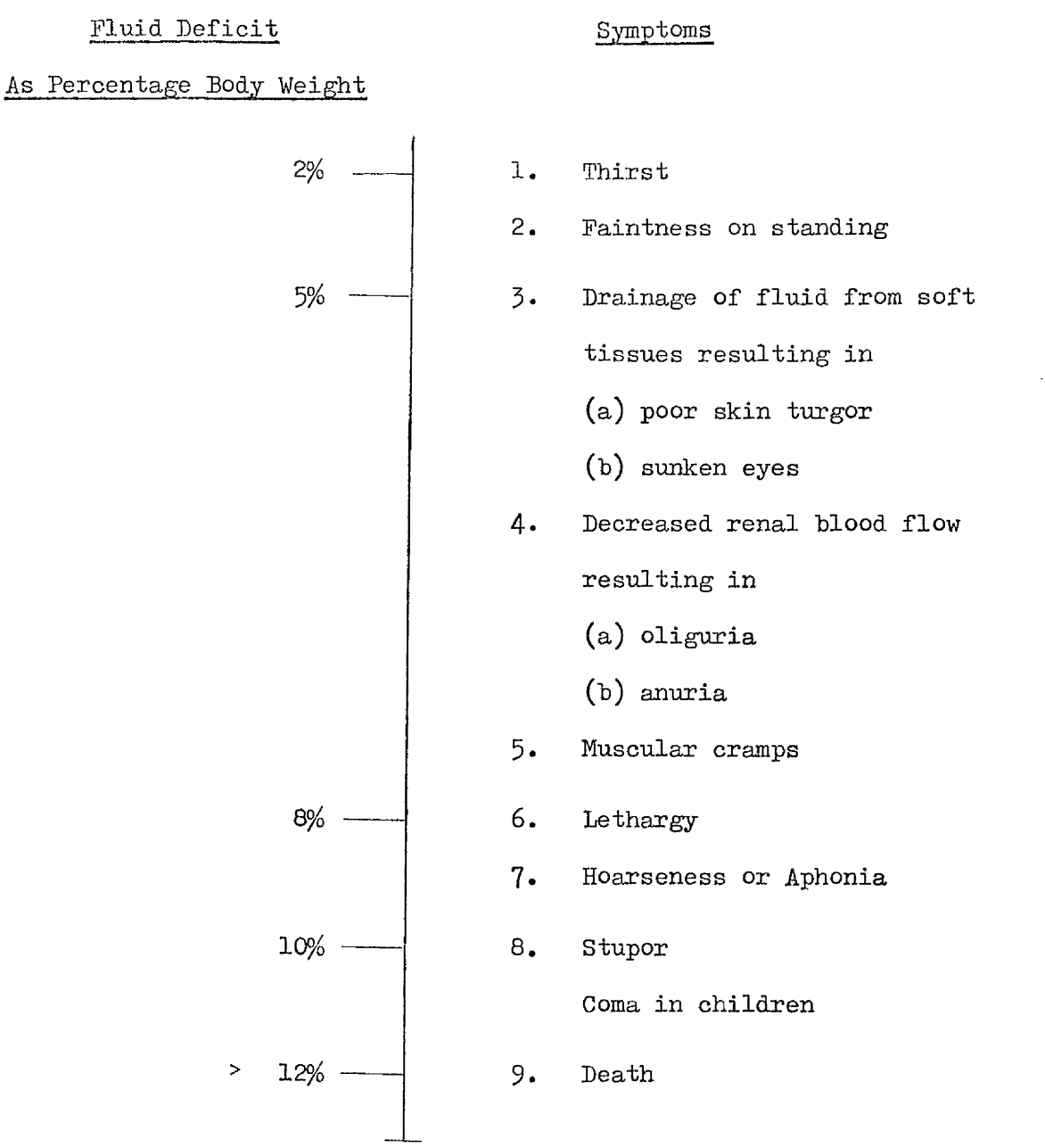
C. Clinical Symptoms

Cholera has an incubation period varying from 24 hr to 5 days (Oseasohn et al, 1966; Hornick et al, 1971). Vomiting occurs in most patients, beginning shortly after the onset of severe diarrhoea. As the disease progresses fluid loss in the stool exceeds the rate of reabsorption by the colon, resulting in an overall deficit of isotonic fluid in the body. Serious isotonic fluid deficit may develop in as little as 4 hr but 1-2 days may be required for the range of cholera symptoms, characterised briefly in Fig 3, to be demonstrated. Pollitzer (1959), Pierce and Mondal (1974) and Mahalanabis, Watten and Wallace (1974) comprehensively reviewed the clinical aspects of cholera.

D. Treatment

Methods of treatment of cholera were reviewed by Hirschhorn

Figure 3. Symptoms of cholera in relation to the amount of fluid loss



et al (1974), and outlined by Frost (1976). Since the symptoms of cholera are entirely the result of water and electrolyte loss from the soft tissues and the blood into the gut, replacement of this fluid can remedy the ill-effects. Fluid replacement therapy reduces the mortality rate from 60 percent in severe, untreated cholera to less than one percent.

The severely-ill cholera patient is given a rapid intravenous transfusion of isotonic fluid, according to his state of dehydration, and is subsequently maintained so that the amount of fluid administered is equivalent to the amount lost. Where the infection is less severe the patient may be protected from dehydration by oral administration of electrolyte solution. Glucose is usually included in such solutions; inclusion of 2% glucose in oral fluid increases the absorptive capacity of the small intestine (Taylor et al, 1968). A typical solution for oral rehydration of cholera patients was suggested by Frost (1976) to be - glucose, 20.0 g; NaCl, 4.2 g; $NaHCO_3$, 4.0 g; KCl, 1.8 g; dissolved in 1 litre of drinking water.

III. VIBRIO CHOLERAE TOXINS

1. Types of Toxin

From his studies on the etiology of cholera, Koch, in 1884, suggested that the disease was an intoxication, since V. cholerae was localised in the lumen of the intestine. The precise nature of the toxic activity remained obscure for many years until publications such as those of De (1959) and De, Ghose and Sen (1960) on the enterotoxicity of V. cholerae culture filtrates aroused fresh interest in the subject. As information on cholera toxins accumulated, Burrows (1968) saw the need for a classification capable of differentiating the various toxic activities. He divided cholera toxins into 3 groups by heat stability

and dialyzability, thus:-

	Heat-stable at 100°C	dialyzable through cellophane
Type 1 Toxins (endotoxin)	yes	no
Type 2 Toxins (protein exotoxin)	no	no
Type 3 Toxins (non-protein exotoxin)	yes	yes

A. Endotoxin

V. cholerae, like other Gram-negative organisms, possesses an outer envelope containing LPS (endotoxin). The LPS is a heat-stable moiety and is contained within Burrows' toxin type 1 group. Pant (1968) isolated LPS and found it was responsible for the "O" antigenicity of vibrio cells. However, the relationship of LPS to the pathogenesis of the organism, if any, is obscured by the rapid action of V. cholerae enterotoxin. Iwert, Leitch and Burrows (1967), found that intra-ileal loop inoculations of V. cholerae in the rabbit increased the levels of serum glutamic-oxalacetic transaminase and lactic dehydrogenase. These increases reflected a type 1 vibrio toxaemia, lethal for mice (Burrows, 1968), and were compared also by Iwert et al to those observed during Gram-negative septicaemias in humans, and in experimental endotoxic shock in rabbits and dogs. In 1968 Iwert et al compared the levels of these enzymes in cholera patients, during the first five days of illness, to those found in normal persons. There were significantly higher levels of these enzymes in the sera of cholera patients. Elevated enzyme levels in rabbits were thought to be due to increased permeability of the lower ileum, allowing absorption of endotoxin or Boivin antigen. Whether humans respond similarly is not known.

B. Heat-Stable Exotoxin

The heat-stable exotoxin, or type 3 toxin according to Burrows' classification, is found in V. cholerae culture supernates, and is both heat-stable and dialysable through cellophane membranes. Studies by Fuhrman and Fuhrman (1960), Huber and Phillips (1960) and Fuhrman, Fuhrman and Burrows (1962) demonstrated the ability of V. cholerae culture supernates to inhibit the active transport of the sodium ion across the skin and bladder of amphibia. The latter workers suggested that inhibition of sodium transport in the intestine during cholera could account for the diarrhoeagenic symptoms of the disease. It was calculated that a three per cent inhibition would result in fluid loss of the same magnitude as that found in the natural infection. However, Grady et al (1968) showed that there was no relation between the enteric toxicity of cholera culture filtrates and the substance causing inhibition of the "sodium pump" in the frog skin. They found a similar inhibition by supernates from a number of ammonia-producing bacteria; inhibition was a linear function of the $\log_{10}$ (NH_3^o) in the preparations. However removal of ammonia from cholera culture filtrates by dialysis (Leitch and Burrows, 1968) left behind a non-dialysable, thermolabile component, which stimulated fluid accumulation, a characteristic of cholera infection, in rabbit intestines.

Thus the heat-stable exotoxin of V. cholerae, demonstrated to be dissolved ammonia, could be separated from the heat-labile exotoxin associated with the production of fluid imbalance during cholera (Burrows, 1968).

C. Heat-Labile Exotoxin (Exo-enterotoxin, Choleragen, Permeability Factor)

The heat-labile exotoxin, or type 2 toxin according to Burrows'

classification, of V. cholerae is considered to be predominantly responsible for the pathogenesis of cholera. This particular cholera toxin is referred to in the literature under a number of terms (Finkelstein, 1973) including "exo-enterotoxin", implying its action on the intestine, and "choleragen". During this review the term "enterotoxin" will be used except in discussion of the intradermal activity of the toxin, where "permeability factor" will be employed; the two terms are synonymous.

Craig (1965) showed that intracutaneous injection, into guinea-pigs or rabbits, of small volumes of stools from bacteriologically-confirmed cholera cases, and from cell-free culture filtrates of V. cholerae, gave identical reactions of erythema and induration 6 to 8 hr after injection. No such reaction was found on injection of live vibrios. The activity of both the stool and culture filtrate preparation was completely abolished by heating at 56°C for 30 min, was decreased by 25-50% after dialysis against 0.85% NaCl for 24 hr at 5°C, but was not affected with trypsin (100 μg/ml) for 15 min at 37°C and pH 7.0. Through these experiments Craig established the production of enterotoxin by V. cholerae both in natural infections and during in vitro culture. Following this observation the production and properties of enterotoxin were studied in an effort to determine its role in human cholera.

2. Production of Enterotoxin

Richardson (1969) demonstrated that enterotoxin could be isolated from culture supernates before any lysis of vibrio cells was apparent. Maximum accumulation in culture supernates occurred during a transitional period between the exponential and stationary phases of growth. The total yield of enterotoxin was shown to be proportional to the length of the linear growth phase.

Aerobic conditions were found to be optimal for production of enterotoxin. Craig (1966) recommended the use of Roux bottles, containing a volume of culture medium which would give a surface to volume ratio of 2 cm^2/ml. Other workers such as Mosley, Aziz and Ahmed (1970) used 100 ml shaking cultures in 3 litre Erlenmeyer flasks, to achieve sufficient aeration. Finkelstein and LoSpalluto (1969) successfully produced bulk toxin in aerated fermenter cultures.

Craig (1966) specified an incubation temperature of 30°C for 48 hr for enterotoxin production; however various alternatives have been equally successful. Coleman et al (1968) grew cultures at 37°C for 6-7 hr, thus avoiding the contamination of the culture supernates with vibrio endotoxin, which was commonly found in older cultures. Richardson, Evans and Feeley (1970) used incubation temperatures of 25-30°C for up to 24 hr.

The optimum pH for enterotoxin production varies with the strain of vibrio. Evans and Richardson (1968) noted the pH 6.6 required for enterotoxin production by strain VC-12, compared to that of pH 7.2-7.8 required by strain 569B (Kaur, Burrows and Cercavski, 1969). The latter workers noted that while no enterotoxin was produced by strain 569B in acid conditions, a non-toxic antigen was still formed in normal or greater amounts. The rate at which enterotoxin is produced is dependent also on the strain of vibrio. Kusama and Craig (1970) incubated two vibrio strains, 569B and B1307, at 30°C and 37°C, and found that enterotoxin was produced in greater amounts at 30°C with both organisms. However, with strain 569B the enterotoxin titre reached a peak, at 24 hr at 30°C, which was maintained for 96 hr. In contrast, the enterotoxin titre produced by strain B1307 declined and was almost undetectable after 24 hr at either temperature.

The enterotoxin moieties produced by the various vibrio strains, despite strain differences in production requirements, are immunochemically very similar, if not identical (Holmgren, Lönroth and Ouchterlony, 1971).

A variety of media have been used for vibrio culture and toxin production. These fall into three broad groups:-

(i) Peptone Media

Peptone water has been used by a number of workers; for example Craig (1965) used a 5% solution, and Evans and Richardson (1968) reduced the peptone concentration to 3% and found that this was adequate for a good yield of enterotoxin. A 3% solution of dialysate of bacto-peptone was found by Coleman et al (1968) to support good growth and a high yield of toxin. This had the advantage that unaltered constituents of the medium could be removed from the choleragen in the culture supernate by dialysis (through a 10k daltons molecular weight exclusion membrane).

(ii) Semisynthetic Media

Finkelstein et al (1966) used a sucrose-inorganic salt medium supplemented with a 1% solution of casamino acids, "Syncase Medium." This medium was modified first by Richardson (1969), who used lactate instead of sucrose, 0.005% yeast extract and a mixture of trace elements in 0.01M Tris buffer, and secondly by Richardson et al (1970) by the addition of potassium and phosphate, substitution of glycerol for lactate, and the use of Tris-maleate buffer, "TRY Medium."

(iii) Synthetic Media

Craig (1966) was the first to use a chemically defined medium, which contained $(NH_4)Cl$, $MgCl_2$, $FeCl_3$ and sucrose, as carbon and energy source, for enterotoxin production. Richardson et al (1970) also

described "TAY Medium" which differed from their "TRY Medium"; the casamino acid component was replaced by glutamate, serine, aspartate and arginine. Callahan, Ryder and Richardson (1971) demonstrated that phosphate and potassium were required also, for maximum enterotoxin production when the casamino acid supplement was removed. This improved medium was designated "TF Medium."

From the literature, none of the above has emerged as the medium of choice. The medium selected by different research groups has largely been governed by the purification steps, if any, to be applied to the culture supernate.

3. Purification of Enterotoxin

Various methods of purification of enterotoxin have been outlined. These include the initial steps of concentration of enterotoxin from crude culture supernates by filtration through Amicon membranes (Duhamel, Talbot and Grady, 1970), or a variety of other concentration-partial purification steps, which may be summarised as follows:-

(a) precipitation with 90% saturated $(NH_4)_2SO_4$ (Finkelstein and LoSpalluto, 1969) which yielded up to 80% recovery of active enterotoxin

(b) absorption to aluminium compound gels (Spyrides and Feeley, 1970), which allowed considerable concentration and purification

(c) dextran sulphate precipitation (Richardson and Evans, 1968) which permitted a 250-fold concentration of enterotoxin contained in culture supernates

(d) ethanol fractionation at $-20^{\circ}C$ (Heckly and Wolochow, 1970), 44% ethanol was sufficient to precipitate enterotoxin.

In addition to these initial steps, further purification was

achieved by either gel filtration or ion-exchange chromatography. These techniques were used in a variety of combinations in an effort to obtain pure enterotoxin. Richardson and Noftle (1970) used dextran sulphate precipitation, followed by $(NH_4)_2SO_4$ precipitation to free enterotoxin from the dextran sulphate. Enterotoxin was separated from contaminating LPS, dextran sulphate and $(NH_4)_2SO_4$ by gel filtration on a P150 polyacrylamide column. Finally it was separated from other impurities by ion-exchange chromatography on DEAE Sephadex A-25. Alternatively, Finkelstein and LoSpalluto (1970) used a series of membrane filtration steps to concentrate their crude toxin preparations. Purification was achieved with Sephadex G-75 and Biogel A-5M columns (Plate 3).

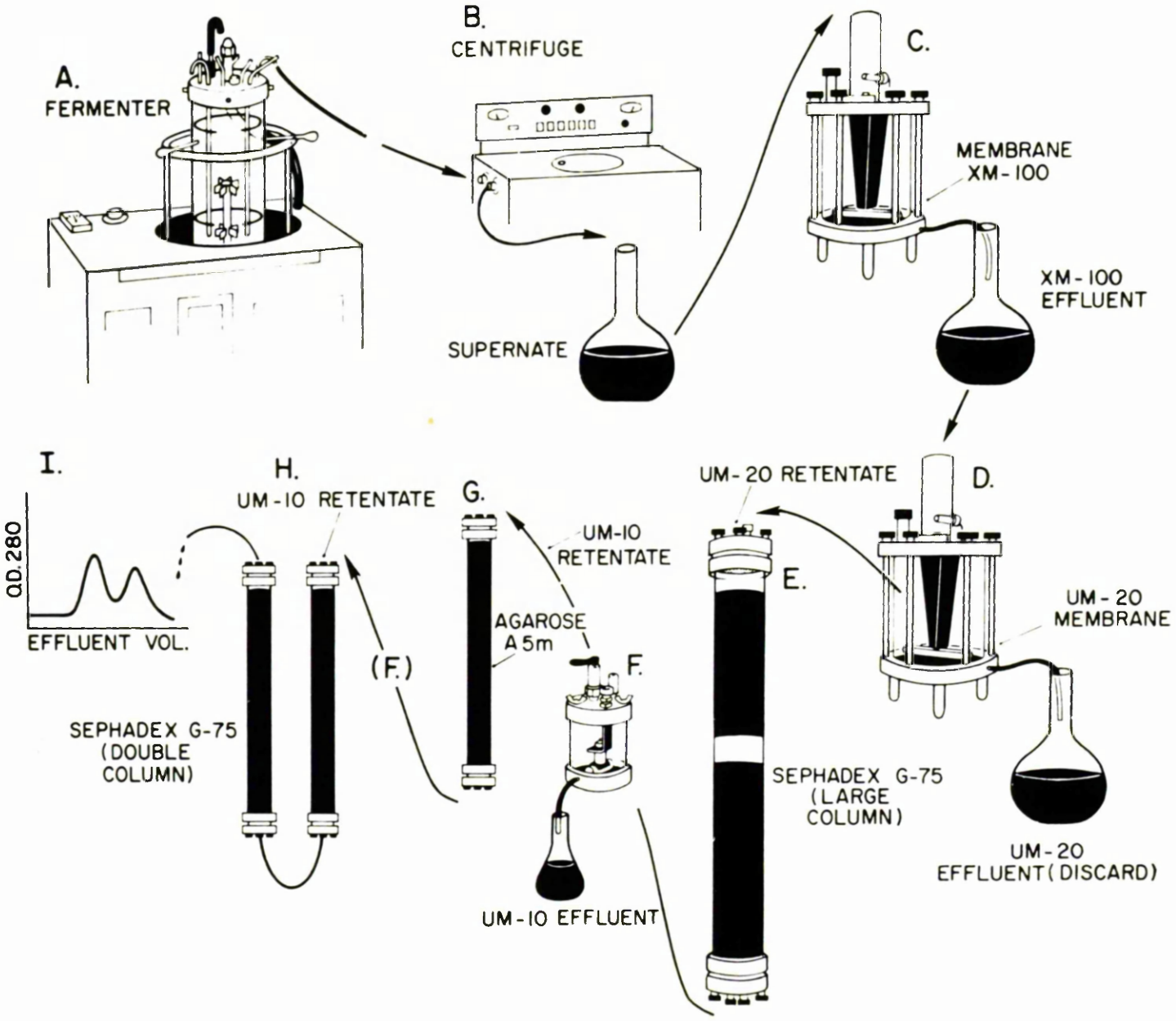
With the growing interest in cholera enterotoxin, both as an immunogen and as a membrane-active reagent, it became desirable to purify it in large quantities. Lewis, Richardson and Sheridan (1976) developed a method involving concentration by membrane filtration, cation exchange chromatography and preparative gel electrophoresis, which could be applied to 6-8 litre cultures.

4. Chemical and Physical Properties of Enterotoxin

A. Composition

With improved purification techniques it was possible to study the properties of enterotoxin. Coleman *et al* (1968) studied its elution profile from DEAE Sephadex and its sedimentation rate in the analytical centrifuge and suggested that it was a lipoprotein with some carbohydrate. However, Kaur *et al* (1969) detected heterogeneity in the preparation used by former workers, and found that most of the lipid could be removed without affecting toxicity (Kaur *et al*, 1970). Heckly

Plate 3. Some of the separation techniques used in the purification of cholera toxin.
(Finkelstein & LoSpalluto, 1970. J. infect. Dis. 121, 565).


A.
FERMENTER
B.
CENTRIFUGE
SUPERNATE
C.
MEMBRANE
XM-100
XM-100
EFFLUENT
D.
UM-20 RETENTATE
UM-20
MEMBRANE
UM-20
EFFLUENT (DISCARD)
E.
SEPHADEX G-75
(LARGE
COLUMN)
F.
(F.)
G.
UM-10
RETENTATE
AGAROSE
A 5m
UM-10 EFFLUENT
H.
UM-10 RETENTATE
SEPHADEX G-75
(DOUBLE
COLUMN)
I.
O.D. 280
EFFLUENT VOL.

and Wolochow (1970) partly substantiated this observation by demonstrating that toxicity was not affected by treatment with either ficin or pancreatic lipase. According to Finkelstein and LoSpalluto (1969) and LaSpalluto and Finkelstein (1972), highly purified enterotoxin contains only protein, with no detectable lipid or hexose.

B. Stability

Craig (1966) was the first to examine the stability of enterotoxin. Toxicity was abolished by heating at 56°C for 30 min; dialysis of enterotoxin at 5°C for 24 hr against 0.85% NaCl caused a 25-50% decrease in toxicity. Exposure to trypsin at 100 μg/ml for 15 min at 37°C at pH 7.0, had no detectable effect on toxicity. Enterotoxin was unstable below pH 6.5 and above pH 9.0, but at neutral pH, although still labile, could be stabilised by 0.01M phosphate buffer, or by a protective colloid such as gelatin (Mosley et al, 1970a). Van Heyningen et al (1971) reported that the toxic activity was inactivated by a water-soluble lipid, extracted from intestinal and other tissues, which had the properties of a ganglioside. This observation may be explained by more recent studies on the binding of enterotoxin to biological membranes (see p. 38).

It has generally been observed that as the purity of enterotoxin is increased, the stability decreases. For example, crude preparations of enterotoxin, such as ultrasonic lysates of agar-grown vibrios, are stable for up to two years in the freeze-dried form when stored under nitrogen at 4°C. However, the estimated half-life of the purified material ranges between 12 hr (Grady and Chang, 1970) and 18 hr (Kaur et al, 1969). Duhamel et al (1970) made the startling comment that purification of enterotoxin did not cause a significant increase

in specific activity, since the increase in purity was offset by a further loss of toxic activity. According to these workers, after passage through Sephadex G-150, only 10% of the total activity of a crude preparation could be recovered, and this figure was reduced to around 1% if further purification steps were employed.

C. Naturally Formed Toxoid - "Choleragenoid"

The mechanism whereby the activity of enterotoxin diminished, began to be understood when an antigenically identical but nontoxic molecule, choleragenoid, was isolated both by Kaur et al (1969) and by Finkelstein and LoSpalluto (1969) by a series of gel filtration and fractionation steps. The purification procedures employed by both of these groups, although different, were capable of resolving the toxin "peak", obtained by previous workers (Coleman et al, 1968), into three and two peaks respectively. In each study, one of these peaks contained enterotoxin, while another contained a nontoxic entity, which appeared to be antigenically identical to enterotoxin, and for which Finkelstein and LoSpalluto (1969) suggested the term "choleragenoid."

It became apparent that higher levels of one of these molecular species were accompanied by lower levels of the other. As the enterotoxin level in a preparation decreased, so the choleragenoid level increased. Callahan et al (1971) demonstrated that the relative proportions of the two substances in culture supernates was dependent on the growth medium. Finkelstein, Peterson and LoSpalluto (1971) found, using I^{131}-labelled enterotoxin, that an increase in the rate of replacement of labelled toxin by labelled choleragenoid resulted from an increase in agitation of the V. cholerae culture, regardless of aeration, under typical cultural incubation conditions.

The latter authors concluded that choleragenoid was neither a protoxin nor a catabolite of enterotoxin, but was a spontaneous degradation product of the enterotoxin molecule.

D. Charge

There was some controversy over the isoelectric point (pI) of enterotoxin since values ranging from below pH 6.0 (Heckley and Wolochow, 1970), pH 6.6 (Finkelstein and LoSpalluto, 1970) to pH 7.0 and even pH 9.0 (Holmgren et al, 1971) were noted. However, this inconsistency may be explained by the observation of Kaur et al (1969) that ammonium sulphate precipitated enterotoxin required an electrolyte for elution from DEAE Sephadex, suggesting that it was positively-charged. Thus, whereas purified enterotoxin preparations were characterised by a negative charge, in all probability this charge was modified when the toxic moiety was complexed with another substance.

In the studies of Finkelstein and LoSpalluto (1970) highly purified enterotoxin was found to have a pI of 6.6. With similarly purified choleragenoid, it appeared that the preparation was not homogeneous in pI. Three similarly-sized, but differently charged proteins were electrofocused; a major species had a pI of pH 7.75 and two minor species focused around pH 7.58.

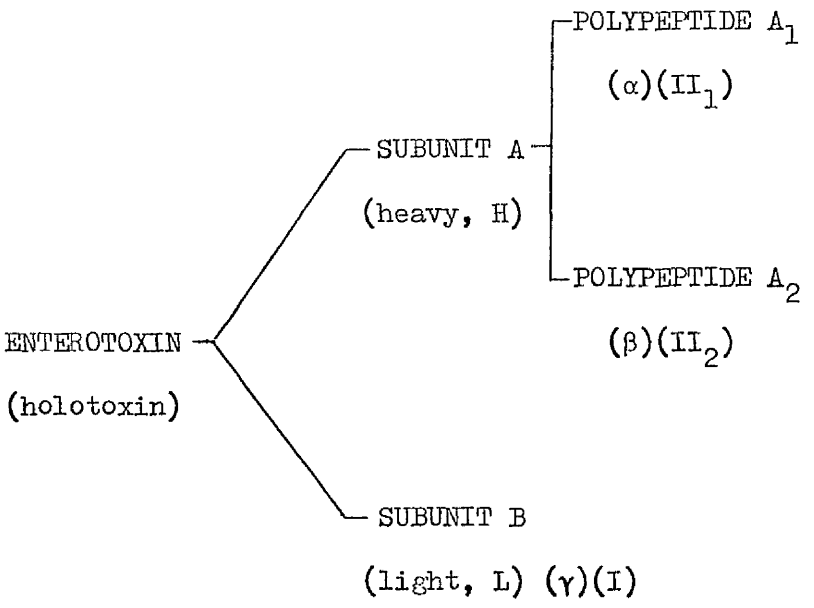
Similarly Roda et al (1977) found that purified enterotoxin was heterogeneous on polyacrylamide gel electrophoresis. Three forms, I (5%), II (15%) and III (80%), were present which were similar in amino acid content and molecular weight, and differed only in net charge. This difference was related to the amide content of the three forms. Deamidation of accessible asparagine and glutamine residues was thought to occur mostly on the B subunit and to result in the conversion of the native toxin, III, to forms II and I via a non-reversible reaction.

E. Molecular Weight and Structure

The rapidly expanding volume of information on the molecular weight and structure of enterotoxin is, unfortunately, confused due to the introduction of a variety of nomenclature systems. In an effort to clarify the following account, Figure 4 is constructed to show the breakdown of enterotoxin into subunits, and to state the synonymous terms by which these subunits are known. The subunits of enterotoxin will be termed A and B throughout this review, except where the work of particular authors is being discussed.

The earlier work on molecular weight determination of enterotoxin was plagued by variability in values, depending on the technique employed. The values ranged from a few thousand daltons to nearly a hundred thousand daltons. At the lower end of the scale values of 12k - 14k daltons were reported by Coleman et al (1968), based on the comparative rates of migration on thin-layer gel filtration of lysozyme and enterotoxin. Finkelstein and LoSpalluto (1969) obtained a value of less than 20k daltons, although Holmgren et al (1971), suggested the slightly higher figure of 25k - 38k daltons; both studies used filtration through Agarose A-5M. Values of around 60k daltons were reported by (a) Finkelstein and LoSpalluto (1969), using filtration through Sephadex G-75, (b) Heckly and Wolochow (1970), using analytical ultracentrifugation, (c) Duhamel et al (1970), using Sephadex G-150 filtration, and (d) Holmgren et al (1971), using Sephadex G-75 filtration. Finally, LoSpalluto and Finkelstein (1972), studying highly purified enterotoxin, calculated its molecular weight to be 82k daltons, using sedimentation velocity diffusion, and 84k daltons, according to meniscus depletion studies.

Figure 4. The various nomenclature systems used to describe cholera toxin subunits



Nomenclature	References
A_1, A_2, B	(a) Van Heyningen (1976) (b) Lai et al (1976) (c) Ohtomo et al (1976)
H, L	Holmgren and Lönroth (1975)
α,β,γ	Kurosky et al (1976)
II_1, II_2, I	Søattler et al (1975)

As the polymeric nature of enterotoxin became apparent, it was possible to explain the range of molecular weights. The extent of polymerisation or dissociation depended on the treatment to which the subunits were exposed. On heating (Finkelstein et al, 1971b), the enterotoxin monomers formed even larger molecular weight aggregates, with a sedimentation coefficient around 30S. These aggregates possessed low levels of toxic activity, but were highly immunogenic in mice (Fujita and Finkelstein, 1972). Finkelstein et al (1971b) used the term "procholeragenoid" for this material, and envisaged that it was an intermediate in the formation of choleragenoid from enterotoxin.

About this time the monomers comprising enterotoxin began to be investigated. Finkelstein et al (1971a) suggested that both enterotoxin (∿ 90k daltons) and choleragenoid (∿ 60k daltons) could be split irreversibly into similarly-sized subunits of approximately 15k daltons. However, in a later publication, Finkelstein et al (1974) presented results which suggested that two distinct subfractions were obtained on filtration of enterotoxin through Bio-Gel P-60 under dissociating conditions in acid urea. The first fraction eluted from the gel, subunit A, was unique to enterotoxin, while the second fraction, subunit B, was present in both enterotoxin and choleragenoid. Isolated A and B fractions gave a reaction of non-identity, against antiserum prepared against enterotoxin, in an Ouchterlony gel diffusion plate. Neither subunit was significantly toxic in experimental animals. It was suggested that subunit B was responsible for the binding to host-cell receptors, while subunit A was thought to be responsible for the activation of adenyl cyclase by enterotoxin. Subunit B was non-covalently associated with subunit A, which was dissociated into two parts by treatment with thiol reagents.

These results were complemented by those of Holmgren and Lönroth (1975) who, by sodium dodecyl sulphate polyacrylamide electrophoresis, identified two types of subunit in the enterotoxin molecule - one of approximately 28k daltons and the other approximately 8k daltons, termed H (heavy, i.e. A) and L (light, i.e. B) subunits respectively. The H subunit was further degraded into two components by reduction and alkylation and it was suggested to be a disulphide bridged dimer. The results suggested that enterotoxin was composed of 7L + 1H subunits, while choleragenoid was composed entirely of aggregated L chains.

Holmgren and Lönroth (1975) indicated also that enterotoxin possessed both enterotoxin-specific antigenic determinants and determinants shared with choleragenoid. The choleragenoid-shared determinants were present in the L-type subunit, while the H-type subunit contained the specific determinants. In the same study, it was demonstrated that enterotoxin and choleragenoid shared the ability to attach to the ganglioside Gm_1 (see p. 38), which suggested that the L subunits were responsible for binding.

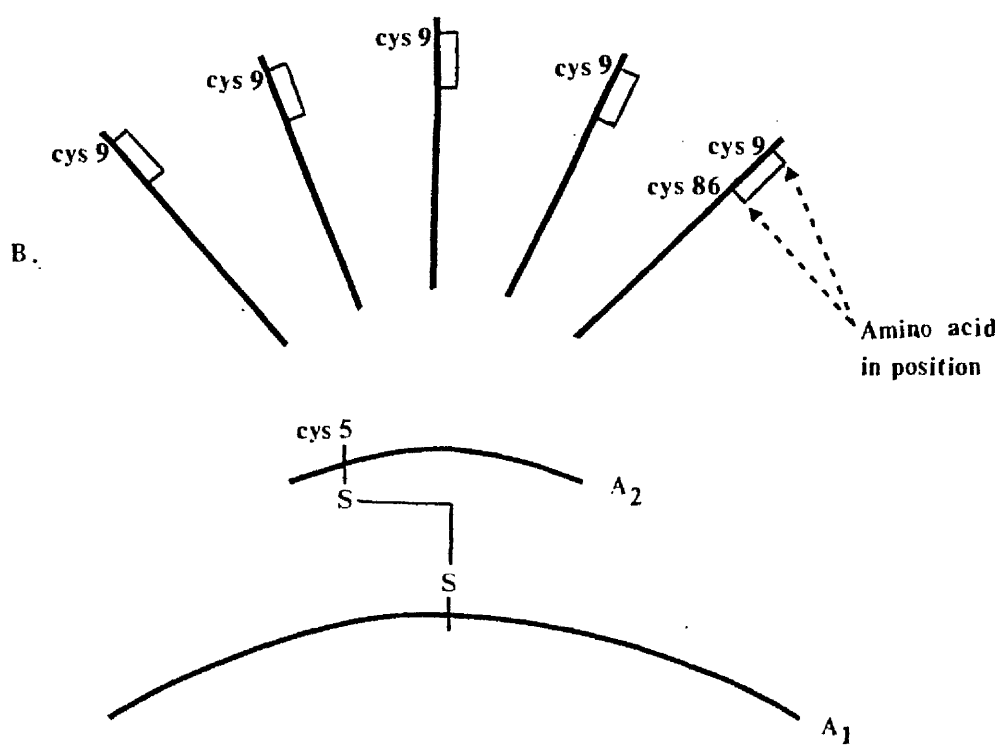
Van Heyningen (1976) stated that enterotoxin was composed of two types of subunit, A and B. Subunit A could be split into two polypeptide chains and was unique to enterotoxin. Subunit B, however, was common to enterotoxin and choleragenoid. It was suggested that subunit B, by binding enterotoxin to the Gm_1-ganglioside receptor molecule in the host cell membrane, facilitated the interaction of subunit A with the adenyl cyclase system.

Although it is generally agreed that enterotoxin can be broken down into the subunits described by Van Heyningen (1976), the precise molecular weights of these subunits remain to be determined. Recent estimations of the molecular weights of these species are summarised below.

Subunit	A_1	A_2	B	Reference
	27	8	8	Cuatrecasas, Parikh and Hollenberg (1973)
	25	7	10	Sattler et al (1975)
molecular	23-24	5.5	10.6	Gill (1976)
weight	24	9.7	9.7	Kurosky et al (1976)
(k daltons)	20	7.5	9.5	Lai, Kendez and Chang (1976)
	23	2.5	11	Ohtomo et al (1976)
	22	5	14	Van Heyningen (1976)
	21.3	7.3	10	Klapper, Finkelstein and Capra (1976)

Having isolated the subunits of cholera enterotoxin, studies of their primary structure began. Jacobs, Niall and Sharp (1974) reported the 20 amino terminal residues of each subunit. Nakashima et al (1976) presented the entire sequence of 103 amino acids of the B subunit, as did Lai (1977). Klapper et al (1976) elucidated the amino terminal sequences of all three subunits.

The secondary structure of enterotoxin was studied by Mendez, Lai and Wodnar-Filipowicz (1975), Lai (1977) and Klapper et al (1977). The latter workers produced the following diagram on the basis of their finding on di-sulphide bridging:-



The position of the cysteine residue in the A_1 was unknown, but was located at position 5 in the A_2 chain. Each of the B chains had an identical amino acid sequence and a single disulphide loop beginning at position 9. Lai et al (1977) suggested the di-sulphide bridge in the B subunit to be between cysteine 9 and cysteine 86.

The exact orientation of the three subunits still poses a problem. An "AB_4" configuration was suggested by Jacobs et al (1974), Van Heyningen (1976) and Kurosky et al (1976). Holmgren and Lönroth (1975), however, suggested that 6 or 7 B chains were associated with the A chains, and Lai et al (1976) agreed on 6 B polypeptides to every "A_1, A_2" complex. The most recent reports by Lai (1977) and Klapper et al (1976) have put the B:A ratio at 5:1.

Gill (1976) proposed that the B subunits were arranged in a ring with A on the axis. A_2 was suggested to hold A_1 onto the B-ring. When examined by electron microscopy (Ohtomo et al, 1976) cholera enterotoxin molecules were found to be of uniform size and arranged in V, Y or ring forms. Roda et al (1977) thought it possible that charge differences among enterotoxin molecules, brought about by deamidation, could account for the difference in conformation noted by the previous workers.

F. Antigenicity, Receptor Binding and Toxic Properties

Kimberg et al (1971) and Sharpe and Hynie (1971) demonstrated that enterotoxin markedly enhanced the activity of the membrane bound enzyme, adenyl cyclase, which catalyses the conversion of ATP to cyclic AMP. It was also found to have a depressive effect on Na-K-dependent adenosine triphosphatase in the intestinal mucosal cell. The net effect was an increase in cAMP concentration in the mucosal cell which

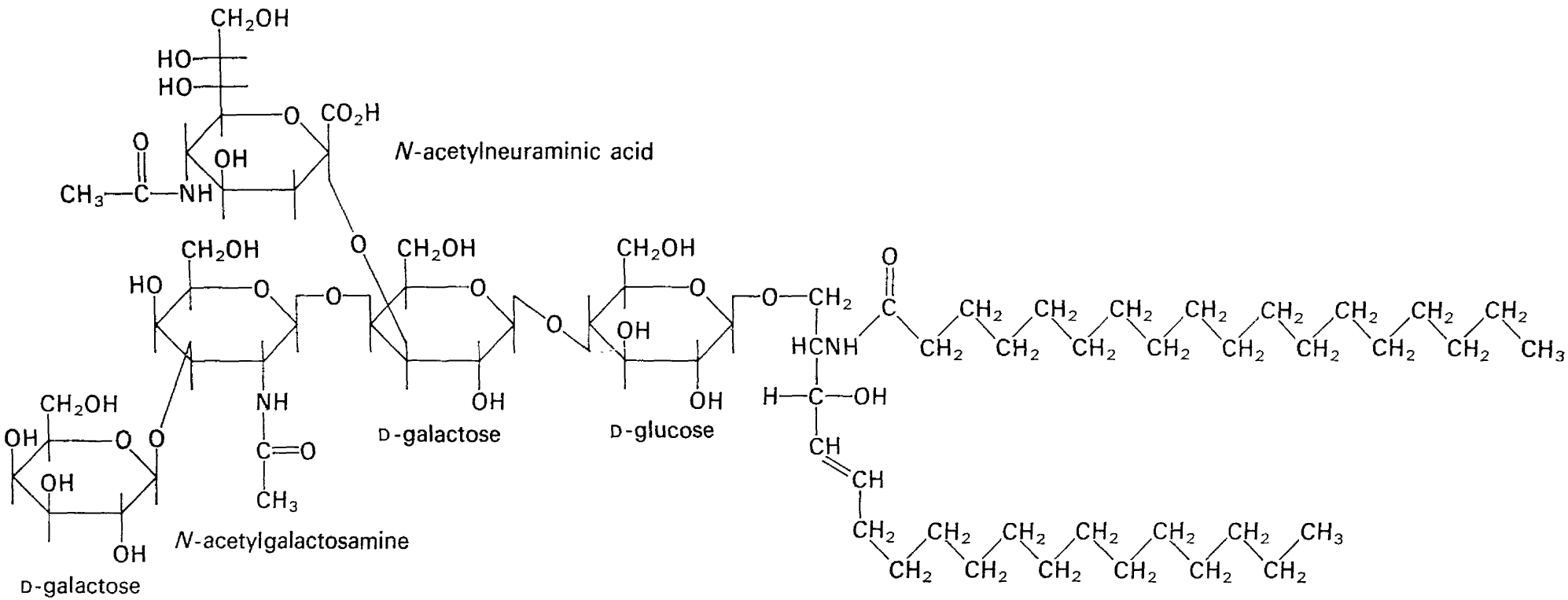
unbalanced the normal ion transport processes, closely dependent on ATP levels. The net efflux of ions from the mucosal cell into the lumen of the intestine, with a concomitant outflow of water from the cell, was judged to be directly responsible for the diarrhoeal effects induced by enterotoxin.

As mentioned already (p. 34) the A subunit was thought to be responsible for the interference with the adenyl cyclase system, while the B subunit was associated with the binding of enterotoxin to the cell surface. Cuatrecasas (1973a, b, c, d), while studying the binding of enterotoxin and choleragenoid to liver and fat cell membranes found that binding to cell membranes could effectively be inhibited by concentrations as low as 1 ng of the monosialoganglioside, Gm_1 (galactosyl-11-acetylgalactosaminyl [N-acetylneuraminyl] galactosyl-glucosyl ceramide) (Plate 4). That this ganglioside was the membrane receptor for cholera enterotoxin was suggested by the work of Van Heyningen (1974a), Van Heyningen (1974b), Hollenberg et al (1974), Holmgren et al (1975a) and Moss, Manganiello and Fishman (1977).

This Gm_1 ganglioside was spontaneously incorporated into cell membranes and thereby greatly increased the sensitivity of the cell to enterotoxin: for example, increased the rate of lipolysis in fat cells due to an increase in cAMP concentration. It also became evident that sensitive cells preincubated in the presence of choleragenoid were no longer influenced by exposure to active enterotoxin. The conclusion drawn from this work was that enterotoxin and choleragenoid both interacted with the same receptor molecule on the cell surface, namely a ganglioside.

Van Heyningen (1974a) noted that treatment of isolated epithelial cells with sialidase, which is secreted by V. cholerae,

Plate 4. The structure of monosialoganglioside, Gm_1.
(Van Heyningen, 1977. Biol. Rev. 52, 517).


N-acetylneuraminic acid
D-galactose
D-glucose
N-acetylgalactosamine
D-galactose
Hydrophilic portion
sugars
Hydrophobic portion
ceramide

increased their ability to bind enterotoxin, since sialidase-sensitive membrane gangliosides lost sialic acid residues to become Gm_1. Although V. cholerae sialidase was active in vitro, Holmgren et al (1975a) found that it had very little effect on the number of toxin binding sites on the intestinal cells in vivo, and did not alter the sensitivity of the small bowel.

Cuatrecasas (1973) noted a lag phase of up to 30 min after the addition of active enterotoxin to sensitive cells, before the appearance of toxic effects. It was therefore suggested that the original enterotoxin- and choleragenoid-receptor complexes were very similar, but only the enterotoxin-receptor complex was capable of undergoing a subsequent rearrangement during the lag phase, within the structure of the membrane, which converted it to a biologically active form.

Contrary to the above reports which implicated Gm_1 as the membrane receptor for cholera enterotoxin, Kanfer, Carter and Katsen (1976) suggested that Gm_3 (N-acetylneuraminylgalactosylglucosylceramide) was the major ganglioside involved in binding enterotoxin to fat cells. Van Heyningen, Van Heyningen and King (1976) suggested that the receptor may be more complex than Gm_1 alone since they showed that enterotoxin binding did not increase proportionally with the amount of Gm_1 incorporated into pigeon erythrocytes, and that the resulting cAMP response did not increase in proportion to the extra toxin bound. It would seem that the function of the receptor may be dependent on additional properties of the surrounding membrane.

Lönnroth and Holmgren (1974, 1975) and Holmgren and Lönnroth (1976) described the effect of chemical modification of enterotoxin on its various properties. Reagents which decreased the ability of enterotoxin to bind to the Gm_1 ganglioside-receptor, caused a correspond-

ing decrease in toxicity. A close association was found between destruction of the ganglioside binding capacity and the antigenic properties of enterotoxin. However, there was also a group of protein modifying reagents, e.g. cyclohexanedione, which abolished the toxicity of the molecule, but had no effect on its receptor binding or antigenic properties - it was suggested that such reagents might prove to be useful in the production of toxoids for immunisation procedures. These results were in agreement with the proposal that ganglioside-binding and most of the antigenic properties of enterotoxin were located on the B subunits, while the toxic activity was associated with another part of the molecule, the A subunit. They also indicated that enterotoxin must be bound to the cell membrane via the B subunit, before the A subunit could become active.

Lönnroth and Holmgren (1975) suggested that lysine residues were involved in the binding of enterotoxin to the acidic Gm_1 receptor, since amino-group reactive substances were particularly effective in decreasing its binding capacity. Similarly, substances which did not affect binding or the aggregation state of enterotoxin but which decreased toxic activity, were found to be arginine-specific. The involvement of arginine residues at the "toxic site" was therefore suggested.

Recently the kinetics of toxin binding and the interaction between the two subunit types required to produce the toxic effect have been studied in detail. For example Holmgren and Lönnroth (1976) found that enterotoxin binding to cells was apparently not temperature dependent, since equal amounts of I^{125}-labelled choleragenoid bound to thymus cells at $0^{\circ}C$ and at $37^{\circ}C$. However, after the addition of unlabelled enterotoxin and choleragenoid it became obvious that binding

at 0°C was a largely reversible process, whereas irreversible binding required incubation at 37°C. It was apparent from the work of Craig and Cuatrecasas (1975) that even after irreversible binding of enterotoxin to the cell membrane, activation of adenyl cyclase was not certain. Their results indicated that partial inhibition of adenyl cyclase activation by anti-enterotoxin IgG could still occur after incubation with toxin for 30 min at 37°C. It was suggested, therefore, that only some of the cell bound toxin formed irreversibly active complexes with the cyclase at the same time.

The events which occur during the lag phase, between enterotoxin-binding and the activation of adenyl cyclase, are still obscure. However, the work of Craig and Cuatrecasas (1975) and of Sedlacek et al (1976) have been illuminating. The former workers studied the interaction of fluorescein-labelled enterotoxin and receptor gangliosides in lymphocyte cell membranes. The latter workers incorporated a synthetic ganglioside carrying a DANSyl-fluorescent label into lymphocyte cell membranes and observed the resulting interaction after the addition of choleragenoid. Similar observations were made on the binding of enterotoxin/choleragenoid to the receptor molecule, in both cases. The receptor molecules which were originally distributed evenly across the cell membrane were seen to move together to form patches or caps (seen as areas of fluorescence). This phenomenon was apparently temperature-dependent since the number of cap-forming cells, on incubation at 0°C, was greatly exceeded by that at 37°C (Sedlacek et al, 1976). This observation, therefore, raises the question as to whether cap formation constitutes the irreversible step in enterotoxin binding.

The mechanism involved in the lateral movement of enterotoxin receptor complexes across the membrane to form caps is entirely open to

speculation at the moment. The capping process was found to be inhibited by substances, such as colchicine, which are known to be inhibitors of the microtubule-microfilament system (Craig and Cuatrecasas, 1975). Although these colchicine-binding, submembrane proteins are thought to be involved in translocation of surface proteins, it appears unlikely that gangliosides are directly linked to submembrane proteins since the hydrophobic portion of their structure, being only 3 nm in length, is too short to span the membrane. Similarly, the polar group of the ganglioside is judged to be too small to contain more than one toxin-binding site, so that cross-linking of gangliosides via toxin molecules is equally unlikely.

Craig and Cuatrecasas (1975) considered that enterotoxin was at least bivalent and on these grounds suggested a possible capping mechanism. They suggested a specific association between erythrocyte ghost glycolipids and membrane proteins, and envisaged a similar effect with Gm_1 ganglioside molecules. If this were so, a membrane protein with several associated ganglioside receptors would act as a single multivalent entity for toxin binding, and would allow lattice formation. They proposed that antitoxin could interfere with this process by reducing the effective valency of cell bound enterotoxin, thus preventing the rearrangement of ganglioside molecules within the membrane, which seemed to be necessary for adenyl cyclase activation.

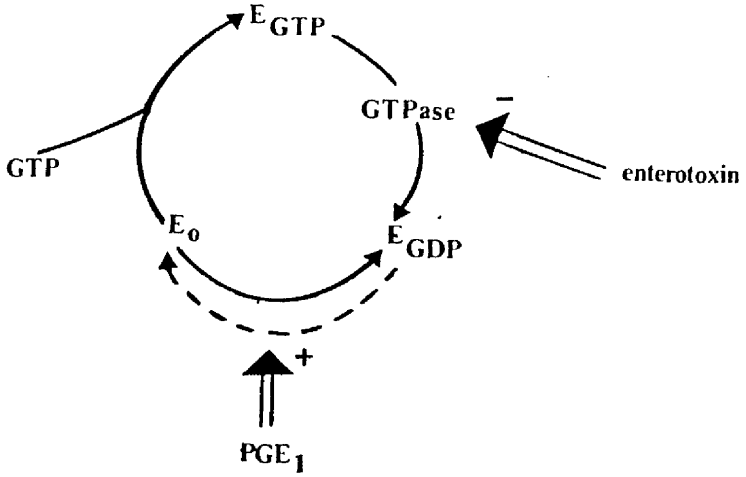
However, it was also pointed out by Craig and Cuatrecasas (1975) that choleragenoid, which was unable to stimulate adenyl cyclase, was as efficient as enterotoxin in production of capping. It was therefore obvious that receptor cross-linking and rearrangement were not sufficient to initiate enzyme activation. Instead, the release of the relatively hydrophobic subunit A into the membrane and its subsequent interaction

with adenyl cyclase was suggested as the remaining step essential for enzyme activation. Such an interaction between subunit A and adenyl cyclase seems probable from the results of Bennet, O'Keefe and Cuatrecasas (1975). Gel filtration of solubilized, stimulated adenyl cyclase demonstrated the co-migration of the enzyme with the "active" 36k dalton enterotoxin subunit. It was also found that enterotoxin stimulated adenyl cyclase could be immunoprecipitated with either anti-enterotoxin or anti-"active" subunit antibodies. Berkenbile and Delaney (1976) studied solubilised adenyl cyclase and also favoured the association of subunit A with the enzyme. However, stimulation of adenyl cyclase was unaffected by solubilisation in detergent when the entire enterotoxin molecule was present. Since the low level of adenyl cyclase stimulation produced after incubation of rat liver plasma membrane with isolated A subunits (also previously shown by Van Heyningen and King, 1975) was completely abolished in the presence of detergent, the authors suggested that some biological modification of subunit A might occur prior to enzyme activation by cell-bound enterotoxin.

Bennet and Cuatrecasas (1975), Craig and Cuatrecasas (1976) and Bennet et al (1976) discussed in detail the events preceding the activation of adenyl cyclase. The exact mechanism of enzyme activation, however, is still not fully understood.

Cholera enterotoxin and several hormones have the ability to increase intracellular levels of cAMP (Berry, 1975). This suggested that enterotoxin and such hormones may have a similar mode of action. Ledley et al (1976) demonstrated a degree of sequence similarity between the peptide chains of enterotoxin and several hormones known to activate adenyl cyclase and suggested that this may account for the similar metabolic effects of these proteins.

Levinson and Blume (1977) showed that, at least in the case of Prostaglandin E_1 (PGE_1), the hormone need not have the same site of action to produce the same effect, i.e. accumulation of cAMP. These workers presented the following model for guanine nucleotide regulation of adenyl cyclase(E) :-



When GTP is bound to the guanine nucleotide regulatory site of adenyl cyclase, the enzyme (E_{GTP}) is in a state of high catalytic activity. When this site is unoccupied (E_o) or filled with GDP (E_{GDP}) the enzyme has little or no activity. GDP functions as an inhibitor by blocking the binding of the activator nucleotide (GTP). Thus the properties of the enterotoxin activated enzyme are due to the decrease in the conversion of $E_{GTP} \rightarrow E_{GDP}$, which normally occurs rapidly via a divalent cation-dependent GTPase. Secondary enzyme regulators such as PGE_1 exert their influence on a different regulatory process, i.e. the affinity of GDP for the nucleotide regulatory site on adenyl cyclase.

It was found by both Parkinson et al (1972) and Grady, Keusch and Deschner (1975) that adenyl cyclase situated in the basal and lateral membranes of the brush border could be stimulated by enterotoxin bound to the mucosal surface. It was suggested that the stimulatory

process did not take place entirely within the membrane since the enterotoxin remains localised on the mucosal surface. Indeed it has been shown by several workers that components of the cell cytosol are essential for enzyme activation. Van Heyningen et al (1976) indicated that NAD, an unidentified macromolecular factor, and glutathione, which possibly facilitated the separation of A_1 and A_2 peptides, were essential for enzyme activation. Flores and Sharp (1976) implicated NAD and an unidentified cytosol component and noted the stimulatory effect of guanyl nucleotide and certain nonhydrolysable analogues of guanosine triphosphate on adenylate cyclase activity. These workers suggested the possibility, later elaborated by Levinson and Blume (1977), that enterotoxin acted on the guanyl nucleotide regulatory protein of the adenyl cyclase complex.

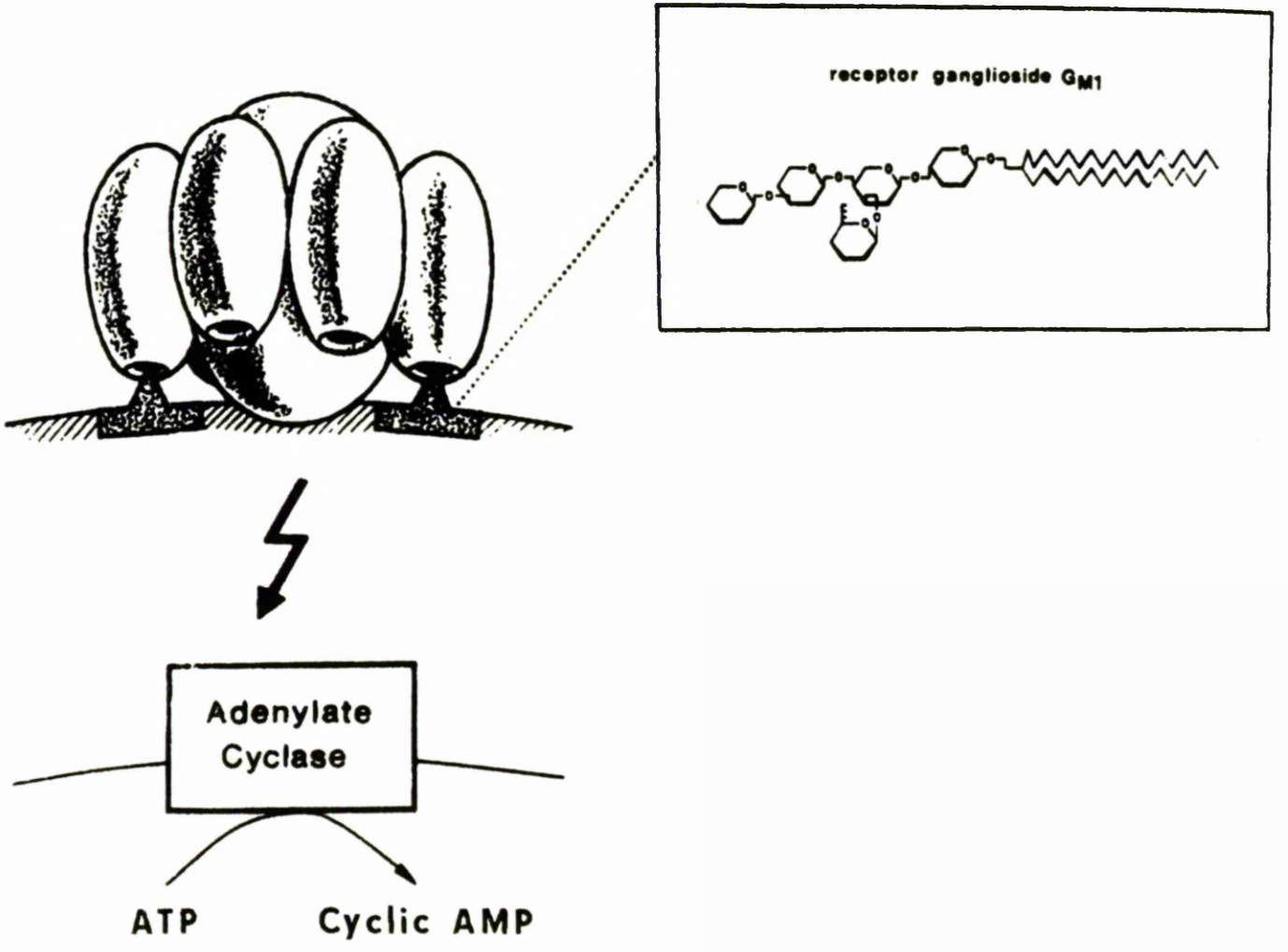
Further confirmatory evidence of the requirement for NAD in enzyme activation was presented by Johnson and Bourne (1977) and Martin, Housley and Kennedy (1977). Moss and Vaughan (1977) found that enterotoxin catalyses the conversion of NAD to ADP-ribose and nicotinamide, and that the production of nicotinamide was greatly enhanced by the presence of L-arginine methyl ester. These workers proposed, therefore, that enterotoxin-induced activation of adenyl cyclase involved the ADP-ribosylation of arginine, or a similar amino acid residue, in an acceptor protein.

The identity of the ADP-ribosylated protein has not been determined, however the GTPase converting $E_{GTP} \rightarrow E_{GDP}$ in the system of Levinson and Blume (1977) offers one possibility.

As outlined by Holmgren and Lindholm (1976) (Plate 5) it is generally accepted that enterotoxin causes a build up of cAMP in the mucosal cell and this is thought to result in increased fluid secretion.

Plate 5. A diagramatic representation of the cholera enterotoxin molecule and its cellular action.
(Holmgren & Lindholm, 1976).

The B subunits bind the toxin to the Gm_1 ganglioside receptors on the cell surface. After a "lag" period the A subunit activates adenylate cyclase, resulting in increased cell production of cyclic AMP.


receptor ganglioside G_{M1}
Adenylate Cyclase
ATP
Cyclic AMP

However, Wu et al (1976) found in immunised animals, that despite a suppression in the fluid secretion response there was still a maximum stimulation of mucosal cell adenyl cyclase. A small fraction of the intracellular cAMP pool was thought to be responsible for fluid secretion, and immunisation might prevent either the generation or mobilisation of this pool. The authors also suggested that their results could be explained if only a small population of the mucosal cells was responsible for the secretion. Thus protection of these cells from enterotoxin action could prevent secretion, although nearly maximum stimulation of the total mucosal adenyl cyclase might be observed.

Field (1976) suggested that only crypt cells were active in secretion (see p. 16), and constituted slightly less than 50% of total epithelial cell mass. Since 40% of the crypt cells are non-proliferative and secretory they would constitute less than 20% of the total epithelial cells. Thus changes in the cAMP content of these cells might not be obvious from measurements on whole mucosa.

5. Biological Properties of Enterotoxin

A. Ileal Loop

Several biological properties of enterotoxin have been discovered which can be used for detection and assay. Among the most useful of these is its ability to induce fluid accumulation following injection into the intestine of experimental animals - a reaction which is thought to parallel closely the response of the human intestine to enterotoxin during infection. This fluid accumulation occurs after administration of both viable toxinogenic V. cholerae or active enterotoxin.

Fluid accumulation may be studied by the induction of diarrhoeic disease of infant rabbits (Dutta, Panse and Kulkarni, 1959) or infant mice (Pitkin and Actor, 1972) or by swelling in ligated segments of the intestines of experimental animals (ileal loops). The latter technique was exploited mainly in the adult rabbit (De et al, 1960), although a variety of other species were examined (Basu and Picket, 1969). Among the more useful were the dog ileal loop (Carpenter et al, 1968) and the rat ileal loop (Aziz et al, 1968).

By eliminating, as far as possible, the factors which led to variation in results, this technique was adapted to allow titration of enterotoxin (Burrows and Musteikis, 1966; Kasai and Burrows, 1966). For example, it was noted that the variability in response to toxin in normal rabbits was minimised if 8 to 10 week old animals were used. Leitch and Burrows (1968) studied the area of the rabbit intestine best suited for this titration. The duodenum was most sensitive to exposure to enterotoxin, however the rate of fluid accumulation in this area was not linear during the course of reaction and thus it was considered to be unsuitable. Sensitivity to enterotoxin in the lower ileum was found to exceed that in the upper ileum, while the colon was completely insensitive. This suggested that the lower ileum was the best site for assay. Other recommendations, such as careful washing of the gut segments before use (Aziz et al, 1968) led to an improvement in reproducibility.

This enterotoxin titration technique in its developed form (Kasai and Burrows, 1966) involved injection of the material into ligated segments of the lower ileum and measuring the reaction at a constant time, usually 8 to 12 hr, after inoculation. The degree of reaction is quantified as the ratio of ml accumulated fluid/cm ligated

segment, which, when plotted against the logarithm of the toxin concentration, gives a typical sigmoid dose response curve. The points making up this curve should be averages of the results of at least 4 loops, one in each of 4 animals - the accuracy of the result is not increased usefully by numbers of animals greater than 8. The linear portion of the curve allows interpolation of a 50% effective dose ($E.D._{50}$) - one unit of toxic activity being defined as the $E.D._{50}$.

The ileal loop technique may also be adapted for antitoxin titration (Kasai and Burrows, 1966). Serial dilutions of antiserum are prepared and mixed with three units of a standard toxin preparation, at 37°C for 30 min, before injection. The $E.D._{50}$ is determined, which corresponds to one unit of residual, unneutralised enterotoxin; thus where 2 toxic units have been neutralised. An antitoxic unit is defined as the amount of antibody capable of neutralising one unit of toxin.

B. Permeability Factor (P.F., cholera toxin)

In addition to its enterotoxic properties, cholera toxin produces a number of other effects in tissues with which it does not come into contact during natural infection. Among these is its ability to increase the permeability of surrounding small blood vessels after intradermal injection into experimental animals such as rabbits and guinea-pigs (Craig, 1965). A discrete area of firm swelling, whose diameter is related to the toxin dose, results from this increased permeability. Pontamine sky blue, a dye which binds rapidly to plasma proteins, particularly albumin, has been used extensively to study this reaction, since on intravenous injection the substance is rapidly extravasated at sites of increased permeability. The diameter of the blueing reaction following intradermal injection of toxin is always

slightly smaller than the diameter of visible swelling; both parameters are thought to be equally satisfactory for toxicity titrations. However the increase in permeability, as a zone of blueing, is used to avoid confusion which might arise where cholera toxin preparations are contaminated with endotoxin. Endotoxin would result in redness and induration but have no effect on vascular permeability.

Although the earlier studies of Craig (1965) suggested that the diameter of the blue lesion was linearly related to the logarithm of the toxin dose, in the 5 to 12 mm range, later work (Craig, 1968) indicated that the dose-response was curvilinear. However, within the limits of 6 to 8 mm diameter lesions the curve was found to have only a very gradually increasing slope, which, for practical purposes, may be regarded as a straight line. The toxin dose eliciting a mean diameter of 7 mm in the rabbit is designated one blueing dose (or 1 B.D.). However due to the marked interanimal variation in P.F. sensitivity, which can be up to 16-fold in pairs of rabbits, it is essential that a reference cholera toxin preparation of assigned potency be titrated in every test animal. For assay purposes the distance between the dose response lines elicited by the reference preparation and the test material, at the 7 mm diameter level, may be taken as a reflection of relative potency, providing that the lines are parallel. By carrying out all tests with replicate injections at randomised sites, the combined error of interanimal variation, injection technique and reading is minimised.

A more accurate assay technique has evolved, however, involving the neutralisation of toxic activity. This has the advantage of rendering the skin response specific for cholera toxin by

demonstrating its neutralisation by cholera antitoxin. The use of an antitoxic rather than a toxic unit of reference is also better because of the greater stability of antitoxin preparations in comparison with cholera toxin preparations.

For such toxin-antitoxin titrations, serial dilutions of toxin are mixed with equal volumes of antitoxin, which contain either 1 or 1/3 A.U./ml. The mixtures are incubated at 37°C for 1 hr before intradermal injection in 0.1 ml volumes, thus giving either 1/20 or 1/60 A.U. per injection site. In this way a neutralisation curve may be constructed, which is sigmoid in shape, and the straight line portion of which proves to be highly reproducible. Since a 4 mm diameter of blueing reaction represents the approximate midpoint of the straight line portion, and therefore the most reproducible point, the toxin dose yielding this reaction was arbitrarily designated as the end-point in such titrations.

The limit of blueing (Lb) dose of cholera toxin is defined as, "The Lb dose of the PF is that amount of PF which, when mixed with one unit of antitoxin and injected intradermally into experimental rabbits, evokes, on average, an area of increased vascular permeability 4 mm in diameter, provided the toxin-antitoxin mixture is injected in a total volume of 0.1 ml." The Lb values thus obtained are highly reproducible, variation in titrations being as low as 1.25 fold, which is in favourable contrast to the high degree of variability in response to toxin alone. Where an injection site has received 1/20 or 1/60 A.U. the toxin potency is expressed as the corresponding fraction of the Lb dose, in other words Lb/20 or Lb/60 respectively.

C. Are Enterotoxin and Permeability Factor Identical?

Since the production and purification methods, as well as observations on stability, apply equally to the enterotoxin, and the intradermal permeability factor, it was assumed that the two activities were different properties of one toxic entity. In general, the two activities were inseparable even with highly purified forms of enterotoxin. Two notable exceptions to this generalisation, however, were the studies of Lewis and Freeman (1969), who reported the complete separation of PF and enterotoxin, and of Grady and Chang (1970) who were apparently able to completely isolate enterotoxin, although their PF preparation retained enterotoxin as a contaminant.

More recent evidence has tended to contradict the findings of these latter workers by supporting the conclusion that only one toxin molecule exists. For example, Holmgren et al (1971) found that the toxin active as PF in the intradermal test produced a reaction of identity in double diffusion gels with the toxin causing cholera-like symptoms in experimental animals. This strongly suggested that a single toxin molecule was active in the two different experimental systems. A similar conclusion was reached by Mosley et al (1970a) who titrated three different toxin preparations against a variety of hyperimmune antitoxic sera. In each case identical end points with both skin and ileal loop tests were found. These workers considered that two independent antigens were unlikely to produce almost identical titres, in each of the sera tested, and suggested that the two assay systems were measuring the same antigen-antibody reaction.

Curlin, Mosley and Greenough (1973), in addition to the ileal loop and skin assays, employed the fat cell assay (see p. 54). Although the sensitivity of the three assay systems differed, these workers found that the antibody titres of various sera tested against

several toxin preparations, were very closely related in each of the three assays. Curlin et al (1973) suggested that one toxin molecule could quite conceivably be responsible for these apparently different activities, if each was the result of a common mechanism - that of increasing intracellular cAMP concentrations.

D. Rat Foot Oedema

Finkelstein, Jehl and Goth (1969) found that injection of enterotoxin into the foot pad of the adult rat, resulted in a reaction analogous to that associated with intradermal injection of PF. An increase in vascular permeability in the rat foot resulted in a marked oedema. The extent of the reaction was measured by immersing the foot in mercury and noting the displacement, from which it was noted that the reaction reaches a peak around 24 hr. This experimental system was related to the intestinal toxic activity since it was inhibited by cycloheximide, which also inhibited fluid accumulation in rabbit ileal loops. This technique was also used successfully (Finkelstein and Hollingsworth, 1970) as a means of evaluating the immunogenicity of several potential antitoxic vaccine preparations.

E. Lipase Stimulating Factor

Another biological activity of enterotoxin is its ability to induce the release of glycerol and free fatty-acids from viable fat cells (Greenough, Pierce and Vaughan, 1970). The resultant increase in intracellular cAMP levels on incubation with enterotoxin at 37°C is responsible for the release of these substances. The increase in cAMP concentration results in the stimulation of intracellular lipase activity which in turn leads to the liberation of glycerol and free fatty acid. This lipase-stimulating activity may be abolished by prior

incubation with antitoxic serum, which supports the proposal that there is only one toxic entity. Curlin et al (1973) presented a detailed assay technique for enterotoxin, utilising the lipolytic reaction.

IV. IMMUNOLOGY OF CHOLERA

1. Antibacterial Immunity

A. Antibody Response to Infection

An increase in the serum levels of both vibriocidal and agglutinating antibodies, in response to infection with V. cholerae has been shown by several groups of workers. Sack et al (1966) demonstrated peak antibody titres 8 to 10 days after the onset of symptoms, and a gradual decrease of both vibriocidal and agglutinating titres during the subsequent 6 weeks. After 2 to 7 months, the vibriocidal antibody titres were reduced to their level at the onset of disease, whereas the agglutinating antibody titres remained slightly higher. McCormack et al (1969) reached similar conclusions and stated that the fall in titre to baseline antibody levels was more marked, within 3 months of infection, in children under the age of 4 years, than in an older group of children up to 14 years.

An increase in serum antitoxin levels was also found after cholera infection. Pierce et al (1970) reported that the antitoxic titre in convalescent cholera patients remained elevated for at least 12 to 18 months, as compared with the rapid fall in vibriocidal antibody titres within 3 months.

Woodward (1971) after a study of cholera reinfections in Bangladesh, concluded that the duration of immunity derived from cholera infection was short, especially in persons whose subsequent reinfection

was due to the heterologous organism. He also stated that the risk of reinfection with V. cholerae was probably only slightly less than the risk of initial infection.

B. Antigens Associated with Antibacterial Immunity

The idea of immunisation with whole V. cholerae was introduced as early as 1884 by Ferran, who employed a live cell vaccine. Haffkine (1894) carried out more extensive immunisation studies with what he believed to be a live, attenuated V. cholerae vaccine. His results demonstrated that immunisation with V. cholerae could lead to immunity to infection (cited from Cvjetanović, 1975).

After the field trials of cholera vaccines in Bangladesh and the Philippines, the value of immunisation against cholera was firmly established. With this in mind, many attempts were made to isolate the protective antigen(s) from the vibrio cells with a view to vaccine production. The vaccines used in the field trials were killed whole cell vaccines which elicited both vibriocidal and agglutinating antibodies, but obviously were incapable of inducing production of antitoxin. Studies by Mosley, Benenson and Barui (1968) and Mosley et al (1968b) revealed a correlation between the decrease of cases and the increase of vibriocidal antibody titres; the doubling of the vibriocidal titres after vaccination was associated with a 50% decrease in the case rate, in a cholera endemic region of Bangladesh. There was a linear relationship between vibriocidal and agglutinating antibody titres in sera from acute and convalescent cholera patients, as reported by Barua and Sack (1964) and Sack et al (1966). Thus cell fractions capable of reproducing the effect of the whole cell vaccines in elevating vibriocidal and agglutinating antibody titres would be potentially important as immunogens. This is particularly true since

the potency of whole cell vaccine is dose-dependent, and the dose is limited by the inherent endotoxic properties of the preparation.

Several purified antigens which stimulate the production of agglutinating and vibriocidal antibodies have been reported. Pant and Shrivastava (1960) obtained two fractions of purified polysaccharide, neutral and acidic, from agar grown cholera vibrios by phenol-water extraction followed by fractionation with cetyl-trimethyl-ammonium bromide. These fractions were apparently homogeneous with regard to electrophoretic mobility and Ouchterlony gel diffusion. Antiserum at high dilution (10^{-8}) could precipitate these fractions. Several purified polysaccharides of Ogawa, Inaba, non-cholera and eltor vibrios were prepared by Kaur and Shrivastav (1964), by extraction of cells with 0.5% phenol, followed by acid precipitation, co-precipitation with $CaPO_4$ and cold acidic 8N HCl alcohol. These preparations were found to contain approximately 0.8 to 2.4% total nitrogen and 55 to 70% polysaccharide. They gave a single precipitin band in Ouchterlony gel diffusion tests (Kaur and Shrivastava, 1965).

Similarly Gallut (1965) extracted cell-wall antigens from classical, eltor and non-cholera vibrios, by heating the autolysed vibrio suspensions at 100°C for 2 hr, and subsequently dialysing before chloroform and alcohol precipitation. A polysaccharide fraction was obtained by phenol extraction, and this produced one precipitin band in Ouchterlony gel diffusion tests. Watanabe and Verwey (1965) prepared an LPS fraction from the culture supernate of an eltor Ogawa organism, which was homogeneous in the ultracentrifuge and electrophoretically. Verwey et al (1965) prepared a Boivin antigen-type material, from sonicated Inaba cells, which contained two antigens one of which was heat-labile. Yoshioka et al (1970) reported the isolation of a

fraction, from mildly sonicated eltor vibrios, which was composed mainly of polysaccharide with only a trace of protein, and which was mouse-protective.

Although these various preparations were obtained by different techniques, several similarities emerged. For instance, all the preparations contained heat-stable and non-dialysable polysaccharide (or lipopolysaccharide) analogous to the type I toxin of Burrows' classification. These preparations were capable of stimulating the production of vibriocidal and agglutinating antibodies in either man or experimental animals.

Pittman and Feeley (1963) supported the use of classical cholera vaccines, based on their finding that the mouse protection afforded by five commercial vaccines following challenge with eltor vibrios, was as good as that against the classical cholera vibrio of the same type. A similar, comparable cross-protection between classical and eltor vibrios belonging to the same type was observed by Watanabe and Verwey (1965) when a purified Ogawa LPS was evaluated by the same mouse protection test.

There has, however, been a great deal of controversy during this pandemic concerning the incorporation of both the Ogawa and Inaba types of biotype eltor into cholera vaccines. The evidence obtained, from the preceding study of cell fractions, indicated that the cell-wall antigens, which do not differ between classical and eltor V. cholerae, played a crucial role in human antibacterial immunity. The antigenic determinants, AB(C) of the Ogawa and AC of the Inaba O-specific polysaccharides, seem to be the key antigenic determinants for protection, although the oligosaccharides of each determinant have not been clarified yet. The common antigen, A, of the Ogawa vibrio seems to be weaker

than the common antigen of the Inaba organism in stimulating vibriocidal and agglutinating antibodies in the rabbit and in protecting the mouse against heterologous Inaba vibrio challenge.

On the other hand, the common antigen of the Inaba vibrio, stimulates appreciable levels of antibody and protection against heterologous Ogawa vibrio challenge. This is confirmed by results obtained in field trials of monovalent vaccines. Mosley et al (1970b) found that immunisation with a monovalent Ogawa vaccine did not stimulate the production of protective vibriocidal antibodies against Inaba infection in children in Bangladesh. However during the Philippines field trial an Inaba monovalent vaccine was found to confer cross-protection against Ogawa infection in children of a similar age group to those in the previous report (Philippines Cholera Committee, 1973a). It was suggested that these results supported the conclusion of Shrivastava (1965) that the use of the Inaba vibrio for the manufacture of cholera vaccine should be adequate. However there were reservations about the possibility of monovalent vaccines enhancing antigenic shift, as had been demonstrated by Sack and Miller (1969) in experimental animals.

In addition to the cell-wall antigens containing polysaccharide or LPS, Neoh and Rowley (1970) isolated a protein antigen from the cell-wall of a classical Inaba strain. This antigen was extracted with phenol-water followed by alcohol precipitation. The final protein fraction contained a small amount of carbohydrate; the protein:carbohydrate ratio was 10.7:1.0. The authors found that the antigenic specificities, against which vibriocidal antibodies were directed, were contained in the stable polysaccharide determinants of LPS and in a protein associated with them. The anti-protein antibodies, in terms of

weight of antibody-protein, were at least as effective as the anti-LPS antibodies in the vibriocidal antibody assay system used. This protein component could be isolated from both Inaba and Ogawa strains, which suggested its possible usefulness as a "universal" cholera vaccine, protective against all serological types.

In a subsequent publication Neoh and Rowley (1972) evaluated three types of protective antibody, which corresponded to anti-LPS, anti-protein and anti-choleragen, in the infant mouse system of Ujiiye et al (1968). They showed that antibacterial immunity, against either the LPS or the protein antigen, was achieved with a smaller amount of antibody (approximately 2.0 μg per 50% protective dose) than that required for an antitoxic immunity (more than 40 μg of antibody protein).

2. Antitoxic Immunity

There are various lines of evidence, mainly from studies in experimental animals, which suggest the importance of antitoxic immunity in V. cholerae infection. Finkelstein (1965) showed that antitoxin was capable of neutralising the toxic effect if enterotoxin and antitoxin were mixed before being fed to rabbits. Further studies by Kasai and Burrows (1966) led to the development of the ileal loop method for the titration of cholera antitoxin, since their studies verified the abolition of fluid accumulation in the rabbit intestine if enterotoxin and antitoxin were mixed before injection. This method was useful in the study of serum antitoxic responses either in human beings or experimental animals after immunisation with cholera vaccines, or in the parallel response in man to natural infection (Mosley and Ahmed, 1969).

Such studies only demonstrated that a toxin-antitoxin reaction, capable of neutralising enterotoxin activity, could occur after mixing the two in vitro. Could this neutralisation reaction occur

naturally in the gut? This question was partly answered by Finkelstein and Atthasampunna (1967) who demonstrated that parenteral immunisation with a preparation of enterotoxin induced immunity to ileal loop challenge of rabbits, with either enterotoxin or viable V. cholerae. The enterotoxin preparation used in this work still contained traces of somatic antigen which might have been responsible for protection. However, Finkelstein (1970) stated that the same immunity to challenge was obtained where purified enterotoxin, or choleragenoid, was used to immunise the rabbits, and where a significant antibacterial antibody response was absent.

Later experiments by Pierce, Kaniecki and Northrup (1972) in which dogs were immunised with purified enterotoxin left no doubt that protection against live vibrio challenge could be achieved by a purely antitoxic immune response. Dogs immunised parenterally with purified enterotoxin in an adjuvant mixture had elevated titres of antitoxin in their serum for 18 months, and were protected against challenge with viable V. cholerae for up to 10 months. However, parenteral immunisation with formalinised toxoid or choleragenoid gave lower titres of antitoxin, and protection of only 5 months duration. Also dogs previously immunised with formalinised toxoid were given oral doses of toxoid after antibody titres due to parenteral immunisation had subsided. Although no rise in serum antitoxin occurred, the animals were once again highly protected against challenge with viable V. cholerae. No such protection was achieved after oral administration of toxoid to dogs which had not been previously immunised parenterally.

Other investigations into the value of oral or intraintestinal immunisation procedures in inducing a protective antitoxic immunity in experimental animals were carried out. For example, Bhattacharya, Narayanaswan and Mukerjee (1968) found that after intraintestinal

immunisation of rabbits with an apathogenic eltor strain, and challenge with toxin 10-12 days later in ligated intestinal loops, there was a significant decrease in fluid accumulation compared to loops in control animals.

Also Burrows, Kaur and Cercaviski (1971) and Kaur, Burrows and Furlong (1971) induced immunity to toxin challenge in rabbits immunised with crude enterotoxin by both the parenteral and intra-intestinal routes; the results indicated that the parenteral route was superior. Holmgren et al (1972) found, however, that subcutaneous and intraintestinal immunisation gave approximately the same level of resistance to ileal loop challenge with enterotoxin, at least after two administrations of the antigen. In this capacity enterotoxin was a better immunogen than formalin-induced toxoid and much better than choleragenoid.

In experiments with highly purified, antigenic derivatives of enterotoxin (i.e. choleragenoid, procholeragenoid and formalin toxoid) Fujita and Finkelstein (1972) showed that mice fed single moderate doses of these antigens developed resistance to intraintestinal challenge with live vibrios, or lethal intravenous challenge with enterotoxin. The purity of the antigens used, apart from the formalin toxoid, appeared to rule out antibacterial immunity as an important component in protection of the mice challenged intraintestinally with live vibrios.

There are several indirect lines of evidence which suggest the importance of antitoxic immunity in protection of human beings. For example, studies by Burrows et al (1971) on serum antitoxin levels in asymptomatic cholera carriers indicated that about 80% of bacteriologically positive carriers had antitoxin levels comparable to those found in convalescent cholera patients shortly after recovery.

Martin, Vernon and Mosley (1969) using a skin test with enterotoxin, in an analogous manner to the Schick test for diphtheria, were unable to demonstrate any correlation between results of skin tests and the likelihood of developing cholera in family contacts. Although these results seem to imply that naturally acquired antitoxic immunity plays little or no role in protection against cholera, it is also possible that the levels of antitoxin measured may have been too low to contribute significantly to immunity. The latter possibility correlates with the results of Pierce et al (1972) who found little or no protection of dogs unless their antitoxin titres were at a fairly high level.

The few available results from toxin/toxoid immunisation of human beings include those of Ungar et al (1970) who found that immunisation with a relatively pure enterotoxin preparation stimulated low titres of agglutinins and vibriocidal antibodies, so that the protective effect, if any, of the low levels of antitoxin produced could not be assessed if such a preparation were used in the field. An $AlPO_4$ adjuvant used with this preparation was found to increase titres of agglutinins and vibriocidal antibodies while having no effect on antitoxin levels. The authors showed that parenteral injection of enterotoxin into human beings was well tolerated, and suggested that vaccination should be done with toxoid stabilised with formaldehyde.

One formalinised toxoid of low somatic antigen content was tested in man for antigenicity and safety (Verwey et al, 1972; Hornick et al, 1972). However, unexpected delayed local reactions, apparently due to partial reversion of the toxoid to active enterotoxin at the injection site (Northrup and Chisari, 1972), prevented evaluation of its

antigenicity at adequate dosage levels. Hornick et al (1972) found that immunisation of human volunteers with this product led to protection, at least against severe, potentially life-threatening diarrhoea induced by challenge with viable V. cholerae. This protection could not confidently be ascribed to the antitoxic immunity (a) because of the relatively small number of volunteers and (b) because of the borderline vibriocidal antibody titres stimulated by this product.

3. "Location" of Immunity

During the course of the cholera infection V. cholerae is localised within the intestinal lumen and enterotoxin is capable of penetrating the tissues only as far as the epithelial cells of the intestine. Thus, any protective immunological reaction must also take place within the confines of the intestine. However, it is unclear whether the antibodies involved in such a protective reaction are synthesised locally in the intestine or whether they are transported from the serum. Experiments testing the protection of either experimental animals or human beings during field trials do not generally permit a differentiation between local and systemic immune mechanisms stimulated by active immunisation, since both serum and copro-antibodies may be elicited simultaneously. Ahmed, Bhattacharjee and Mosley (1970) pointed out that serum vibriocidal antibody levels may be correlated with levels of protection, only because they are a direct reflection of the presence of intestinal antibodies.

A. Protective Activity of Serum Antibodies

Evidence that serum antibodies may play an active role in protection against cholera has come from several sources. Finkelstein

and Hollingsowrth (1970), using rat foot oedema as an indicator of biological activity of enterotoxin, found that resistance could be transferred passively by antibody-containing serum from previously-immunised rats. Holmgren et al (1972), using the ileal loop technique, demonstrated that intravenous administration of antitoxin to non-immunised rabbits was capable of conferring some resistance to ileal challenge with enterotoxin - a result which bore more relevance to natural human infection.

Pierce and Reynolds (1974) agreed with the previous observations on the possible protective role of serum antibodies. These workers studied the protective role of antitoxic IgG in dogs transfused with either whole plasma or purified serum IgG from hyperimmunised dogs. They found that IgG antitoxin could be recovered in washings from jejunal loops in non-immunised, plasma-transfused dogs, and also that dogs were protected against intrajejunal challenge with enterotoxin. They were also able to show that dogs transfused with antitoxic IgG were highly protected against orogastric challenge with viable V. cholerae.

B. Protective Activity of Intestinal Antibodies

There is also a substantial amount of evidence which suggests that if an immune response to cholera is to be effective, antibacterial and antitoxic antibodies must be available in the intestine to neutralise an invasion by V. cholerae.

As early as 1947, Burrows, Elliott and Havens suggested the importance of copro antibody in cholera infection, by studying the relation of copro antibody titre and numbers of V. cholerae secreted by experimental animals. Jenkin and Rowley (1960) also predicted the importance of a defence mechanism against V. cholerae, involving antibody and macrophage, in the gut. Mosley and Ahmed (1969) showed

that unless enterotoxin was rapidly neutralised by excess antitoxin present in the intestine, fluid accumulation occurred in ileal loops. Thus under natural conditions it would appear that antitoxin, to be effective, must be present in the intestine to neutralise enterotoxin in the short interval between its release from the vibrio cell and its binding to the epithelial cell membrane.

It is also known that anti-bacterial antibodies in the intestine are capable of decreasing the absorption of vibrios to the intestinal wall of rabbits (Freter, 1970). Schrank and Verwey (1976) showed that the villi and intervillous spaces in the rabbit intestine were covered by a layer of mucoid material, which formed a zone between the intestinal contents and the tips of the villi (see Fig 2). In normal animals the onset of fluid accumulation was concomitant with the establishment of large masses of organisms in the intervillous spaces and crypts of the intestine, after the successful penetration of this mucus zone. However, with animals immunised with a cell-wall antigen of V. cholerae, the bacteria were clumped and restricted to distribution in the lumen and luminal border. These results implied that antibacterial antibodies, if they are to cope with infection, must be present inside the intestine.

Other studies by Kaur et al (1971) and Kaur, McGhee and Burrows (1972) implicated tissue-associated antibodies in immunity to cholera, at least in rabbit ileal loops. The intestinal epithelium of rabbits, immunised either intraperitoneally or intraintestinally, was separated into crypt and villus cell fractions, and the enterotoxin-neutralising antibody content of ultrasonic lysates of these cells was assayed. Antibody was found primarily in the crypt cells, while villus

cells contained only small amounts of antibody later in the immune response. The finding of such tissue-associated antibodies was consistent with the idea that antibodies might be present continuously at the site of infection, in the intestine, without being subject to the enzymic degradation processes going on in this region. The results of Kaur et al (1971) were substantiated by Pierce and Reynolds (1974) who found that protection in dogs, against intrajejunal challenge with enterotoxin, was not diminished by extensive washing of the jejunum prior to challenge.

The preceding reports demonstrated that antibodies produced in the serum in response to immunisation were capable of passing into the intestine and intestinal tissues where they could best afford protection against V. cholerae infection. Recent studies by Pierce and Gowans (1975) suggested that coproantibodies might be produced, in response to immunisation with choleragenoid, independently of serum antibody production. These workers found that dogs could be protected, for up to 8 months, against orogastric challenge with viable V. cholerae, in the absence of a lasting elevated level of serum antitoxin titres. In this case, therefore, protection was not associated with antibodies derived from the serum. This work established that a coproantibody response could be entirely responsible for protection against cholera. However, it is still unknown whether serum antibodies can protect against naturally-acquired cholera. On the one hand there are workers (Pierce and Reynolds, 1974; Schrank and Verwey, 1976) who found that passively administered serum antibodies, either antitoxic or anti-bacterial, were capable of protecting experimental animals against intraintestinal challenge. On the other hand Holmgren et al (1975b)

suggested that, with enterally administered enterotoxin, only locally produced antibodies were protective. The question remains to be resolved.

C. Antibody Classes Involved in Immunity

Over the past few years the classes of antibody produced in response to cholera infection, and their location in the body have been revealed. In general, serum antibodies were IgM in the primary response and IgG in the primary and secondary responses; however, IgA stimulation was noted particularly in association with the intestine.

Merrit and Sack (1970) and Felgner, Bücken and Richter (1975) studied the sensitivity of serum immunoglobulins to 2-mercaptoethanol (2-ME), after natural infection and after cholera vaccination. The former workers examined acute and convalescent cholera sera and found 2-ME sensitive antibodies in acute phase sera, while in convalescent sera and sera from patients demonstrating a secondary immune response the antibodies were mainly 2-ME resistant. The latter workers obtained evidence to support the view that in non-endemic countries 2-ME resistant vibriocidal antibodies reflected antibacterial immunity against cholera, following vaccination, more than did the total vibriocidal activity. In endemic areas Mosley et al (1969b) demonstrated that vibriocidal activity paralleled protective activity. Felgner et al found that 2-ME resistant antibodies persisted no longer than 6-9 months in 50% of vaccinees despite frequent revaccinations at 6-8 monthly intervals.

This work indicated the importance of serum IgG levels in determining resistance to cholera infection. Pierce and Reynolds

(1974) suggested that serum IgG was protective since they found that dogs transfused with IgG antitoxin were highly protected against orogastric challenge with viable V. cholerae. However, Northrup, Bienenstock and Tomasi (1970) found secretory IgA together with significant amounts of IgA material of the size of serum IgA and smaller, as well as IgG and IgM, present as molecular fragments, in cholera stools. They suggested that these immunoglobulin fragments were probably the result of enzymatic degradation of the antibody molecules after their secretion into the gut.

Mostly IgA has been the immunoglobulin found in the stools of cholera patients and in the intestine of experimental animals. Sasaki et al (1970) found IgG and IgM in the sera, and IgA in the intestinal walls and intestinal contents of germfree mice monocontaminated with V. cholerae. Waldman et al (1971) and Waldman et al (1972) studied human cholera patients in an endemic area, and found elevated mean levels of IgG, IgM and most markedly IgE in their sera, compared to the mean levels detected in Europeans. IgA was the predominant class of immunoglobulin in samples from faeces and intestinal contents. The elution pattern from Sephadex G-200 and the association with secretory component was indicative of secretory IgA. The levels of serum IgA rose significantly in cholera patients during convalescence.

Steel, Chaicumpa and Rowley (1974) isolated three classes of antibody from rabbits immunised with V. cholerae. IgG and IgM were isolated from the sera of animals inoculated parenterally, while IgA antibodies in intestinal washings and colostrum were found only where oral immunisation was used. The preparations of IgA contained little complement-mediated vibriocidal or opsonic activity. However when

studied in the infant mouse lethality test this IgA was considered, on the basis of weight, to protect as well as IgG or IgM.

Kaur et al (1972) studied ultrasonic lysates of intestinal epithelial cells of rabbits immunised by the intraperitoneal or intra-intestinal routes. The intracellular antibody appeared to be almost exclusively IgA. Pursuing a similar line of investigation, Pierce and Gowans (1975) found mainly IgA in the antitoxin-containing cells in the lamina propria of rats immunised with choleragenoid.

V. CHOLERA VACCINES

1. Standard Vaccine

The cholera vaccine now in use is a saline suspension containing 8×10^9 killed vibrios/ml. The killing and preserving agent used is commonly phenol, although formaldehyde, organic mercurials (e.g. thiomersal), or mild heat ($56^{\circ}C$ for 30 min) may be employed. The vaccine is bivalent, containing equal numbers of the Ogawa and Inaba serotypes.

Two doses of 0.5 ml and 1.0 ml are given to adults (0.1-0.3 ml for children under the age of 10 years) by the subcutaneous or intra-muscular route. In epidemics, however, single 1.0 ml doses are used for mass immunisation, and may be almost as effective as the two-dose regime in an endemic area.

The immunity induced by this vaccine is of short duration, persisting for no longer than 6 months. The dosage can not be increased since concentrations greater than 8×10^9 cells/ml tend to produce local tenderness, and less frequently lead to a slightly more severe reaction, lasting for several days (Feeley, 1970).

2. Experimental Vaccines

A number of controlled field trials have been done during the present cholera pandemic to study the efficacy of whole cell vaccines. These vaccines differed in content and concentration and the vaccination schedules varied. The studies were made mainly in Bangladesh, where the greatest proportion of cases was due to infection with the Inaba serotype, and in the Philippines where cholera was due almost exclusively to the Ogawa serotype. Cholera is endemic in both of these regions, however in Bangladesh classical and eltor vibrios are equally prevalent, while eltor vibrios are almost entirely responsible for cholera infection in the Philippines. Table 1 outlines the vaccines used in these field trials. The efficacy of the vaccines was judged from the percentage of vaccinees who did not contract the disease within the stated period of observation.

As a result of these studies, a number of important factors, pertinent to cholera vaccination programmes, have come to light:-

"Cholera vaccines prepared from only classical V. cholerae protect against eltor infection"

During the field trials in the Philippines, where eltor vibrios were responsible for infection, it was noted that both classical and eltor bivalent vaccines conferred protection to 50% of vaccinees, for up to two months. The immunity derived from the classical vaccine declined more rapidly than that with the eltor vaccine, which suggested that the use of the strain homologous to the infecting strain was preferable. However, a classical bivalent vaccine incorporated in an oil-adjuvant gave levels of protection above 50% for 6 months at least, in the same trial. Unfortunately this vaccine could not be recommended

Table 1: Cholera vaccine field trials during the years 1963-1971

Area/Date of Field Trial	Vaccine(s) used	References
Bangladesh 1963-64 and 1964-65	(a) Whole cell vaccine of high mouse-protective potency (b) purified Ogawa LPS	Benenson, Joseph & Oseasohn (1968) Benenson et al (1968b) Benenson et al (1968e) Mosley et al (1968a,b); Mosley (1969)
Bangladesh 1966-67	whole cell vaccine of average potency	McCormack et al (1969) Mosley et al (1969a); Mosley et al (1969b) Mosley et al (1972)
Bangladesh 1968-69	(a) monovalent, whole Ogawa cells (b) monovalent, whole Inaba cells (c) purified Inaba antigen	Mosley et al (1970a) Mosley et al (1973)
Philippines 1964	(a) classical bivalent vaccine (b) eltor bivalent vaccine (c) classical bivalent, oil-adjuvant vaccine	Azurin et al (1967) Mosley (1969)
Philippines 1966 & 1967	eltor bivalent vaccine	Philippines Cholera Committee (1968 & 1973b)
Philippines 1970-71	(a) classical monovalent, Ogawa cells (b) eltor monovalent, Ogawa cells (c) classical monovalent, Inaba cells (d) eltor monovalent, Inaba cells	Philippines Cholera Committee (1973a)
Calcutta 1964 & 1965	(a) - (d) classical vaccines (e) eltor vaccine	Das Gupta et al (1967)

for practical use because of severe reactions in approximately 2% of vaccinees (Azurin et al, 1967). As yet no field trial has provided information on the protection derived from eltor vaccines against classical cholera infection.

"The Inaba serotype induces protection against infection with the heterogolous as well as the homologous strain"

When monovalent Ogawa and Inaba whole cell vaccines were used in the Philippines (1970-71), against Ogawa infection, it was found that the Inaba vaccine afforded protection which was not significantly less than that resulting from the Ogawa vaccine. These vaccines protected 60-70% of the vaccinees for up to 7 months, which was surprising (Philippines Cholera Committee, 1973a) since the previously studied bivalent vaccines had offered maximum protection for only 3 months post vaccination. However, in the 0-4 year age group, which is the group at highest risk of infection in endemic areas, the homologous Ogawa vaccine seemed to be more effective than the heterologous Inaba vaccine.

These two vaccines were also used in Bangladesh (1968-69) against Inaba infection. In this case the Inaba vaccine was far superior in eliciting protection (approx 84%) than was the Ogawa vaccine (approx 48%). The Ogawa vaccine failed to protect children under 5 years, and the cross-protection it produced in older children was thought to be due to the boosting of their naturally acquired immunity (Mosley et al, 1973). The latter observation probably explains why the Inaba vaccine was less effective than the Ogawa vaccine in the 0-4 year age group studied in the Philippines.

Sack and Miller (1969) pointed out that monovalent vaccines carry the risk of enhancing antigenic shift in the infecting strain.

This together with the fact that it is advantageous to vaccinate the high-risk under five-year-olds with the homologous strain, precludes the use of a monovalent Inaba vaccine.

It was also pointed out by Sinha and Bhaskaran (1973) that immunity against V. cholerae was strictly serotype specific. Two different serotypes were used for the immunisation of rabbits and for their ileal loop challenge. Cross protection was only evident between Ogawa and Inaba subtypes of serotype I. Bivalent serotype I vaccine was found to confer protection against both subtypes, although immunity to Inaba infection was of shorter duration than to Ogawa infection.

"Older members of a cholera endemic community are immune by virtue of their constant exposure to the cholera antigens"

Throughout the field trials on cholera vaccination, it was noted that the highest infection rates occurred in young children. In older people, however, constant exposure to V. cholerae resulted in the build up of a naturally acquired immunity which was boosted by cholera vaccination. The older group therefore derived greater levels of protection from vaccination.

Azurin et al (1967) found that the vaccines used in the Philippines (1964) produced poor levels of protection in the youngest age group (6 months - 9 years) but fairly high levels in the oldest age group (over 25 years), however, both these groups had higher attack rates than the intermediate age group (10-25 years). Lowering of the body's natural resistance to infection by factors such as poorer general health, probably accounted for the higher infection rate in the over 25 year age group.

"Children are likely to withstand higher dose levels of whole cell cholera vaccine, without adverse reaction, than are their adult counterparts"

Benenson et al (1968a,b) reported results of studies in Bangladesh on a whole cell vaccine of particularly high mouse protective potency (see p. 72). Delayed reactions to this vaccine, although not severe, were noted in adults 4-7 days post vaccination. Children, however, were free of adverse reactions. The fact that children could withstand higher vaccine doses was interesting since, as previously mentioned, this age group probably require a larger dose than adults, for maximum protection.

"Dosage of cholera vaccine administered to adults is of little importance"

The Philippines Cholera Committee (1968) studied the effect of concentration of bivalent eltor cholera vaccine, and the number of applications, on the resultant levels of protection over the following 6 months. Their results are shown below.

vaccine group	number of 1 ml doses	cells per ml of vaccine	No. of vaccinees protected	percentage protection of vaccinees
1	1	8×10^9	45,977	53
2	2	8×10^9	47,712	55
3	1	16×10^9	50,315	58

Although the third group demonstrated the highest protection level, this was not significantly different from that afforded by either of the remaining two vaccination schedules.

In a separate study in parenterally immunised human volunteers Verwey et al (1969) studied the effect of the immunisation schedule on

vibriocidal antibody response to whole cell vaccines. It was found that two initial doses separated by 1 week were most advantageous; if the interval was increased to 4 weeks the response was no better than that obtained by a single administration. Reimmunisation after 6 months yielded vibriocidal titres which were generally lower than those obtained on initial immunisation. The response was not improved by employing two reimmunisation doses.

"Levels of protection in children are increased by multiple doses of cholera vaccine"

In Bangladesh in 1966-67 a cholera vaccine of average potency was tested in 40,000 children between the ages of 3 months and 14 years, in one or two dose schedules. A single dose protected 46%, and two doses, at an interval of 1 month, protected 64% of vaccinees. The single dose was ineffective in children under 5 years of age, but induced significant protection in older children. This protection, however, even with 2 doses, did not extend beyond the first 3 months of an 8 month cholera season. It was also noted that prior administration of vaccine did not affect the clinical course of cholera; thus preexisting antibodies did not reduce the severity of the disease.

This one or two dose schedule was followed by annual injections for 3 years. It was established that increasing protection reached a maximum after 3 doses, as shown below.

number of doses	No. of vaccinees protected	percentage of vaccinees protected
1	4,600	46
2	6,400	64
3	8,100	81
4	7,600	76

Protection was also more sustained after reimmunisation being 50% and 39%, 1 and 2 years respectively after the fourth injection. However in comparison to treatment costs, this was an expensive programme.

"Route of vaccine administration may be important"

Although this subject has not been studied extensively, the Philippines Cholera Committee (1973a,b) noted that both the subcutaneous and intradermal routes of inoculation gave a significant degree of protection against infection. However, with the subcutaneous route, protection was evident for up to 6 months after vaccination, compared to 4 months when the administration was intradermal, and was more effective in children in the 1-5 year age group.

Unfortunately none of the field trials has discovered a cholera vaccine or vaccination programme which will provide lasting levels of protection in all sections of the community, and which is economical enough for widespread use.

VI. PROSPECTIVE CHOLERA VACCINES

1. Vibrio Antigens

A. Cell Fractions

The possibility of cell fractions being effective immunogens against cholera infection was explored in several studies. This work has ranged from the specific treatment of whole vibrio cell suspensions, to the isolation of distinct antigenic moieties from the vibrio cells.

Altieri et al (1970) examined the possibility of preparing an anticholera vaccine by extracting cell suspensions with Genetron-113 (fluorocarbon extraction). In comparison to control vibrio suspensions not treated with Genetron, the experimental vaccine had a lower nitrogen content and was also less toxic to mice. However, it was also less

effective in protecting mice against infection and in inducing agglutinating and vibriocidal antibody responses in rabbits. The authors suggested that this procedure would be useful in the production of cholera vaccines with fairly high antigenic content and yet relatively low toxic capacity. A similar result was obtained by Nagy (1972) who treated whole cell cholera vaccine with sodium deoxycholate. This procedure resulted in a substantial reduction of toxicity with a concomitant reduction in immunising capacity of the vaccine for mice.

Among the earlier investigations of particular protective vibrio cell antigens, were those of Watanabe and Verwey (1965) and Verwey et al (1965) (see p. 7). These studies led to the isolation of a lipopolysaccharide antigen from Ogawa cell supernates, which was protective against homologous but not heterologous challenge in mice (Watanabe and Verwey, 1965) and a Boivin-antigen-like material from Inaba cells which protected mice both against homologous and heterologous challenge (Verwey et al, 1965). These materials were re-examined in experimental animals and human volunteers by Watanabe et al (1969), for immunogenicity compared with whole cell vaccine. The deproteinated Ogawa LPS antigen was protective in mice in less than 0.01 μg amounts, and was therefore considered on a weight basis to be a good antigen. However it was less efficient in stimulating vibriocidal antibody production in human beings. The Inaba protein-LPS complex on the other hand elicited vibriocidal responses which were at least equal in height and duration to those elicited by a whole cell vaccine of equivalent mouse protective activity. Nagy (1972) showed that crude endotoxin extracted from heat-killed vibrios, conferred protection on mice, no less than that conferred by whole cell vaccine. It would thus appear that an effective cholera vaccine could be prepared from the outer envelope layers of V. cholerae.

There has been some interest in the protective capacity of V. cholerae ribosome preparations, and their fractions, as immunogens. Pitkin and Actor (1972) immunised mice with a ribosome-containing fraction (RF) obtained from V. cholerae Ogawa, and studied the passive immunity conferred to the progeny, via the colostrum, of these mice to lethal challenges of Ogawa and Inaba organisms. A 20 μg dose of RF protected 50% of the offspring against the homologous challenge, at 7.5 weeks of age, and significant protection was observed up to 15 weeks of age. The protection achieved against the heterologous serotype, however, was of limited duration. Jensen et al (1972) stated that ribosomal preparations from V. cholerae Ogawa and Inaba were protective immunogens for mice challenged with either serotype. These workers isolated a protective antigen from the ribosomes by column chromatography. This antigen was found to be a heterogeneous colloid containing protein, lipid and carbohydrate in the ratio of 3:1:1 - not unlike the protective antigen of Verwey et al (1965).

B. Enterotoxin Preparations

Whole cell cholera vaccine contains no enterotoxin (Feeley and Roberts, 1969); however, due to its central role in the production of disease, this toxin can not be ignored as a potentially very important immunogen (Finkelstein, 1969). The former workers prepared toxoids by (a) treating culture filtrates with 0.2% formalin at 35°C for 4 days, and (b) by heating the filtrates at 56°C for 45 min. The formol toxoid was found to be 3-5 times more antigenic than the parent toxin, while the heated preparation was less antigenic. Holmgren et al (1972) studied the resistance to challenge with enterotoxin in ileal loops of rabbits immunised with enterotoxin, choleragenoid or formalin-

induced toxoid. They found that both subcutaneous and intraintestinal immunisation stimulated a protective immunity. However in comparison to Feeley and Roberts (1969) these workers found enterotoxin to be a better immunogen than formol toxoid, which in turn exceeded the efficacy of choleragenoid.

Similarly Thomson, Walker and Knight (1972) demonstrated anti-toxic responses in rabbits after immunisation with (a) formol crude toxoid or with (b) crude or purified enterotoxin detoxified by heat (choleragenoid).

Although enterotoxin preparations were shown to be immunogenic, and toxoids which lacked the characteristic biological properties of enterotoxin were found to induce protective immunity, there was a problem in the spontaneous reversion of choleragenoid to active toxin. However, means have been developed whereby the reversion of choleragenoid to the toxic state can be safely abolished, thus making choleragenoid preparations more acceptable for general use. Thomson et al (1972) found that formol toxoids prepared by incubation of purified enterotoxin for 2 days in 0.2% v/v formalin, reverted to the active state in vitro on incubation at 37°C. Immunisation of experimental animals with these toxoid preparations resulted in death after about 6 days, in some cases due to reversion to active enterotoxin. However, data presented by Finkelstein et al (1971b) showed conversion of enterotoxin to choleragenoid after heating at 60°C for 2 hr. Thomson et al demonstrated that such a toxin would remain irreversibly non-toxic. Saletti and Ricci (1974a) used glutaraldehyde to detoxify enterotoxin which had been (a) partially purified with $Al(OH)_3$ only or (b) further purified by Biogel A-5M and Sephadex G-75 filtration.

These toxoids appeared to have no residual toxicity when injected into the skin of rabbits. The partially purified toxoid retained most of the immunising capacity of the native enterotoxin, however the highly purified preparation did not retain immunogenicity in rabbits. These workers (Saletti and Ricci, 1974b) also studied the possibility of detoxifying their partially purified enterotoxin with formaldehyde and glycine. This process appeared to be successful since no histological changes were seen after injection of the toxoid into the skin of monkeys, while appreciable titres of antitoxin were elicited.

Rappport et al (1974) produced a toxoid which was unable to revert to the active form either in vitro or in vivo. Purified toxin was treated with large volumes of gluteraldehyde at pH 7.8, to yield a toxoid capable of producing elevated, prolonged antitoxin in rabbits, and of protecting immunised guinea-pigs from intradermal injection of enterotoxin. After acrylamide gel electrophoresis and gel filtration of this toxoid, a correlation was suggested between irreversible elimination of toxicity and the formation of higher molecular weight, polymeric toxoid molecules.

2. Oral Vaccines

A. Vaccines containing Non-viable Antigens

An ideal cholera vaccine would be one which stimulated prolonged antibody production at the mucosal surface of the intestine. However, results have indicated that oral or enteral vaccination is inferior to parenteral administration of vaccines, and requires multiple applications before a reasonable level of protection is obtained (Holmgren et al, 1975b). Agarwal and Ganguly (1972) worked with heat inactivated lysates of V. cholerae Ogawa L-forms, using the oral route

of administration, and found that 5 biweekly doses were required to produce an adequate antibody response lasting up to 6 weeks. In both of these cases the oral vaccines stimulated copro antibody production.

An interesting advance in oral immunisation was published recently by Pierce and Gowans (1975) who found that a maximum increase in the number of antitoxin-containing cells resulted when enteral immunisation was preceded by a parenteral inoculation two weeks earlier. These workers suggested that the primary inoculation established immunological memory in the secretory IgA system of the intestine, so that subsequent enteral application of choleragenoid acted as a "booster."

B. Vaccines containing Live V. cholerae

To overcome the problem of multiple oral immunisation, the possibility of using a live, avirulent vaccine was studied, so that the multiplication of the vibrios would provide the necessary prolonged antigenic stimulus. Bhattacharya and Mukerjee (1968) and Bhattacharya et al (1968) studied the increase in vibriocidal and antitoxic antibodies respectively, after oral administration of an apathogenic eltor strain to rabbits. A rise in intestinal vibriocidal antibodies, associated with a superior rise in serum vibriocidal antibodies, was found after oral immunisation. The level of these antibodies was maintained by repeated administration of this live vaccine. Where the antitoxic response was concerned, oral immunisation of rabbits was found to stimulate significant protection against ileal loop challenge in these animals with graded doses of enterotoxin, after 10-12 days.

Subsequently this avirulent eltor vaccine was tested in human volunteers (Sanyal and Mukerjee, 1969). After neutralisation of gastric acidity, the vaccine was administered orally to 25 subjects,

and gave significantly increased serum and gut associated vibriocidal antibody titres which were maintained over 3 and 6 month periods of observation of copro-antibody and serum antibody, respectively. No ill effects were noted in any of the volunteers.

Contrary to these findings, Cash et al (1974) found that only 3 out of 32 vaccinees developed a significant rise in vibriocidal or antitoxic titre, two weeks after immunisation with one of two possible live vaccine strains studied. In 5 of the vaccinees the rate of clinical infection after challenge with virulent V. cholerae was no different from that in unvaccinated controls. The reason suggested for this poor result was that, in comparison to the strain used by Sanyal and Mukerjee (1969), these vaccine strains did not remain viable in the intestine long enough to act antigenically.

3. Vaccines with Adjuvants

In the 1964 Philippines field trial (Azurin et al, 1967) a cholera oil-adjuvant vaccine was used with promising results. The vaccine stimulated protection for up to 6 months after vaccination in 50-60% of individuals, which was far in excess of that recorded for the plain vaccines used at the same time. Unfortunately the oil-adjuvant produced severe reactions in a small percentage of the vaccinees, despite having been subjected to the usual safety tests (Ogonuki, Hashizume and Takashashi, 1967) by testing in experimental animals and in a small group of human volunteers. For the most part the only reaction seen in these volunteers was erythema and slight swelling at the site of injection, and in the worst cases the diameter of this reaction was 8 cm or less. Although these results were considered acceptable, the results of the field trial were not so encouraging. Cases of abscess and granuloma

formation at the site of injection were reported about a month after inoculation and continued to be noted for over a year. All the biopsies examined showed granuloma formation with infiltration of lymphocytes, macrophage, plasma cells and frequently epitheloid cells as well as multinucleate giant cells. In some cases there was a marked proliferation of connective tissue and formation of small patchy scars. The softening and liquefaction of necrotic tissue which resulted was associated with marked infiltration of leucocytes. It was suggested by Ogonuki et al that these local changes were due to the combined effects of both foreign body and local hypersensitivity reactions.

Promising results were obtained by Joó, Csizér and Juhász (1972) with adsorbed cholera vaccines ~~at least~~ in experimental animals. These workers assayed the potency, by rise in vibriocidal antibody titres, of whole-cell vaccines adsorbed to either aluminium hydroxide or sodium alginate in comparison to a plain vaccine. They found that in mice, rabbits and monkeys the immune response to the $Al(OH)_3$-adsorbed vaccine significantly exceeded the response to the plain vaccine. The sodium alginate adsorbed vaccine and the plain vaccine did not differ in efficacy in mice. A field study is however required, as was the case with the oil-adjuvant vaccine, to assess the true potential of the $Al(OH)_3$-adsorbed vaccine in future vaccination programmes.

MATERIALS AND METHODS

1. Strain of Vibrio cholerae

Vibrio cholerae NCTC 7254 Inaba serotype, was originally isolated during a cholera epidemic in Egypt in 1947. A sub-culture of this strain was kindly provided by Dr. T.F. Elias-Jones, City Laboratories, Glasgow.

2. Buffers and Diluents

A. Physiological Saline (0.15M)

Sodium chloride (34.0 g) was dissolved in distilled water and the volume made up to 4.0 litres.

B. Phosphate Buffered Saline (P.B.S.) pH 7.2

(0.01M phosphate buffer, 0.15M NaCl)

Solution A: 3.58 g $Na_2HPO_4.2H_2O$ were dissolved in 1.0 litre distilled water.

Solution B: 1.56 g $NaH_2PO_4.2H_2O$ were dissolved in 1.0 litre distilled water.

For use 72.0 ml of solution A and 28.0 ml of solution B were mixed and the volume made up to 200 ml with distilled water to give 0.01M phosphate buffer. Sodium chloride (8.766 g) was added to each litre of phosphate buffer.

C. Borate Gelatin Saline (B.G.S.) pH 7.5

Solution A: 30.91 g boric acid were dissolved in 1.0 litre of distilled water.

Solution B: 87.66 g sodium chloride were dissolved in 1.0 litre of distilled water.

Solution C: 60.0 g gelatin were dissolved in 1.0 litre of distilled water.

Solution D: 4.0 g sodium hydroxide were dissolved in 1.0 litre of distilled water.

For use 100 ml of A, 80 ml of B, 3.3 ml of C and 22 ml of D were mixed. The volume was made up to 1.0 litre with distilled water and the pH was adjusted to 7.5.

D. Veronal Buffered Saline (V.B.S.) 5X

Solution A:	NaCl	85 g	1400 ml distilled H_2O
	Sodium Barbiturate	3.75 g	
Solution B:	Barbituric acid	5.75 g	500 ml hot distilled H_2O
Solution C:	Stock 1.0M $MgCl_2$	20.32 g $MgCl_2.6H_2O$	100 ml distilled H_2O
	3.0M $CaCl_2$	3.33 g $CaCl_2$	

To make 5X concentrated V.B.S., Solution A, Solution B and 5 ml of Solution C are added together and the total volume made to 2 litres. The concentrated buffer is stored at -20°C. Working solutions are prepared freshly before use by diluting the concentrated solution 1:4 in distilled H_2O. The pH of the resulting solution is checked to be pH 7.3-7.4.

3. Media

A. Protease Peptone (2%) pH 7.5

Protease peptone (Difco Laboratories, Michigan, U.S.A., 2.0 g) was dissolved in 100 ml distilled water. The pH was adjusted to 7.5 using sodium hydroxide.

B. Nutrient Agar pH 7.4

Nutrient agar, code CM3 (Oxoid, London; 28 g) was dissolved in 1.0 litre of distilled water by heating at 100°C and sterilised at 121°C for 15 min. The medium was cooled to 50°C, an aliquot was retained to confirm pH and the remainder was dispensed into petri-dishes.

C. Thiosulphate-citrate-bile salt sucrose (T.C.B.S.) Agar pH 8.6

(Becton Dickinson Co. Ltd., Edinburgh)

Constituents: yeast extract, 5.0 g; sodium citrate, 10.0 g; peptone, 10.0 g; sodium thiosulphate, 10.0 g; oxgall (dehydrated), 5.0 g; sodium cholate, 3.0 g; sucrose, 20.0 g; sodium chloride, 10.0 g; ferric citrate, 1.0 g; thymol blue, 0.04 g; bromothymol blue, 0.04 g; agar, 14.0 g.

The powdered T.C.B.S. agar (86.0 g) was evenly suspended in 1.0 litre of distilled water. The suspension was heated, with frequent agitation, before boiling for 1 min. The medium was cooled to 45°-50°C, an aliquot retained to confirm pH, and dispensed into petri-dishes.

D. Gel Diffusion Medium

Ion Agar No. 2 (Oxoid, 15.0 g) and sodium chloride (16.0 g) were added to 1.0 litre of distilled water and steamed for 45 min. Methyl orange (0.12 g) and 50 ml of 10% (w/v) phenol saline solution were added. The medium was filtered through Whatman filter paper (grade 113) and dispensed in 25.0 ml amounts in universal bottles until required.

4. N.I.H. Cholera Toxin

Freeze-dried filtrate from cultures of V. cholerae, strain Bl307 grown in a medium containing 2.0% Bacto-peptone broth (Difco) and 0.5% sodium chloride adjusted to pH 7.3 with sodium hydroxide solution, according to the method of Craig (1966), was obtained from the National Institute of Allergy & Infectious Diseases, Bethesda, Maryland. When reconstituted with diluent on the basis of 40 ml per 1.0 g of material the potency was 5,000 blueing doses (B.D.) per ml as determined by

intradermal injection in guinea-pigs, i.e. 1 mg was equivalent to 200 B.D. The material was also tested in the canine model and caused a cholera-like diarrhoea; 1.0 g was active in the dog intestinal loop and 1-20 mg in the rabbit intestinal loop.

5. Cholera Toxoid

The sample of cholera toxoid VT 2144 B5 was kindly provided by Dr. R.O. Thomson, Wellcome Research Laboratories, Beckenham, Kent.

V. cholerae (Inaba 569B) was grown in fermenters, then killed and detoxified with formalin. The organisms were removed by centrifugation and filtration and the culture filtrate passed down an immunoadsorption column. The toxoid was eluted with acid, the eluate being then neutralised. This purified toxoid then had to be reformolised to prevent reversion to toxin. The method of immunopurification used is described in Hughes, Thomson and Knight (1974), J. applied Bact. 37, 603 using cholera antitoxin in place of tetanus antitoxin. This was incorporated into toxoid vaccines.

6. Purification of Cholera Toxin

A. Ammonium Sulphate Precipitation

N.I.H. cholera toxin (3.0 g) was dissolved in 5.0 ml B.G.S. and dialysed overnight at 4°C against 95 ml of a 100% saturated $(NH_4)_2SO_4$ solution. The contents of the dialysis sac were removed and centrifuged at 8,000 rpm for 30 min to deposit the precipitate. The precipitate was resuspended in a small volume of B.G.S. and dialysed against B.G.S. for 4 days at 4°C. The protein-containing solution (10 mg/ml), C.T.II, was stored at -20°C.

B. Sephadex Gel Filtration

Cholera toxin (10.0 g; N.I.H.) was dissolved in a small volume of B.G.S. and precipitated with 90% saturated $(NH_4)_2SO_4$ at 4°C overnight. The precipitate was isolated by centrifugation at 8,000 rpm for 30 min. The precipitate was resuspended in B.G.S. and dialysed against B.G.S. for 24 hr at 4°C. The toxin-containing solution obtained was concentrated with Carbowax (polyethylene glycol; B.D.H. Chemicals Ltd., Poole, England).

Gel filtration columns contained either Sephadex G-100 or Sephadex G-200 (Pharmacia, Uppsala, Sweden) and were equilibrated with B.G.S. by running several litres of this buffer through the gel. The void volume of each column was found by applying 2 ml of Blue Dextran 2000 (Pharmacia) (2 mg/ml B.G.S.). This material was eluted with B.G.S. The volume of buffer collected from the column before the elution of Blue Dextran was the void volume.

The concentrated toxin solution was applied to the column and run into the gel bed. A few ml of B.G.S. were pipetted onto the column and run into the gel in order to rinse the toxin solution from the wall of the column. The head of B.G.S. buffer was connected to the column and the $E_{280\ nm}$ of the eluate was recorded constantly using a Uvicord ultraviolet absorptiometer and chart recorder (L.K.B., Bromma, Sweden). The eluate was collected in 2.0 ml volumes.

The protein peak containing cholera toxin was determined using the mouse blueing test and Ouchterlony precipitin test against cholera antitoxin. Fractions comprising this peak were pooled and concentrated using Carbowax. The toxin-containing solution obtained (C.T.III) was stored at -20°C.

7. Preparation of Cholera Toxin

V. cholerae NCTC 7254 was inoculated into 10.0 ml of 2.0% protease peptone (Difco Laboratories, Michigan) pH 7.5 and incubated at 37°C for 24 hr. Aliquots of this culture (0.5 ml) were inoculated into 100 ml 2.0% protease peptone containing 1.0% sodium chloride, in 1.0 litre Roux bottles. The bottles were incubated on their sides for 18 hr at 37°C.

Vibrios were removed from these cultures by centrifugation at 6,000 rpm for 30 min. The supernate was filtered through a 0.65 μm cellulose acetate membrane (DAWP 04700; Oxoid, Basingstoke, England), before the addition of $(NH_4)_2SO_4$ to give a final saturation of 90%. Precipitation was allowed to occur overnight at 4°C before removal of the precipitate by centrifugation at 8,000 rpm for 30 min. The precipitate was resuspended in a small volume of B.G.S. and dialysed against B.G.S. for 48 h before concentration with carbowax.

The concentrate was applied to a column of Sephadex G-200 and eluted with B.G.S. Fractions (2.0 ml) were collected and tested for blueing activity in hairless mice. The fractions within the toxin-containing peak were pooled and concentrated with carbowax and subsequently stored at -20°C.

8. Source of Animals

New Zealand White rabbits, Ham 1/Cr mice and hr/hr hairless mice were obtained from the closed colonies maintained in the animal house of the Microbiology department. Dunkin Hartley guinea-pigs were either obtained from the same source or purchased from Porcellus Animal Breeding Ltd., Heathfield, Sussex.

9. Immunisation Procedure

A. Immunisation of Mice

Adult mice (20 per group) were used for the production of antisera. Cholera toxoid or whole cell vaccine, either in saline or an adjuvant mixture (0.1 ml) was injected into each mouse intraperitoneally (i/p). Three weeks after inoculation the mice were sacrificed; blood from 5 animals was pooled and the serum was collected.

B. Immunisation of Guinea-Pigs

Guinea-pigs were used for the production of antiserum and gut tissue extracts, and for the ileal loop test. The guinea-pigs were divided into groups with respect to the nature of the vaccine to be administered.

(i) Toxoid Vaccines

(a) Preparation and Administration of Vaccines

Guinea-pigs 256-279. Single oral dose

In the first series of experiments involving oral administration of cholera toxoid, vaccine mixtures were prepared. The 0.1 ml oral dose was composed of 50 μg cholera toxoid in saline containing 7.5% (w/v) gelatin alone, or together with 250 μg mycobacterial glycopeptide (ST217) or 250 μg *M. tuberculosis* strain DT heat-killed organisms. A similar series of vaccines was prepared with the addition of 25% sterile liquid paraffin.

The measured dose of each vaccine was administered to each of a group of four guinea-pigs, using a 1.0 ml syringe without a needle as an applicator and releasing the mixture at the back of the mouth.

Guinea-pigs 280-301. Single intramuscular dose

The second series of experiments was done using a single intramuscular injection of cholera toxoid in water-in-mineral oil emulsions

containing mycobacterial adjuvant preparations. The 0.1 ml injection dose contained 60 μg cholera toxoid alone, or together with 250 μg of either, one of the glycopeptides ST208, 210, 217, or whole organisms of M. tuberculosis strain DT, suspended in water-in-oil emulsion (0.3 ml saline, 0.2 ml Arlacel A (Atlas Powder Co., Wilkington, Delaware) and 0.5 ml Bayol F (Esso Petroleum Co. Ltd., Purfleet, Essex)).

(b) Comparison of oral immunisation with combined intramuscular (i/m) and oral immunisations

For parenteral immunisation the guinea-pigs were injected with 0.5 ml of vaccine i/m into the hind leg. For oral immunisation gelatin capsules were filled with 0.3 ml of vaccine. Guinea-pigs were divided into equal groups and treated according to the following immunisation schedules:

Primary Immunisation: Animals were immunised either orally or i/m. Four weeks later the blood and small intestines were removed from one group of guinea-pigs, and the ileal loop test was done in another group.

Primary Immunisation plus one oral Booster: Six weeks after the primary inoculation a secondary oral booster dose was given to each guinea-pig. Two weeks later groups of animals were tested as described above.

Primary Immunisation plus two oral Boosters: Four weeks after the secondary dose a tertiary oral booster dose was given to each animal. Two weeks later groups of animals were tested as described above.

The vaccines given to each group of animals were:-

Guinea-pigs 303-320. Primary oral, i/m, or combined oral/i/m dose, followed by an oral booster dose

A water-in-oil emulsion containing 2.0 mg cholera toxoid in 3.5 ml saline, 1.0 ml Arlacel A, 2.5 ml Bayol F and 2.5 ml n-Octadecane

($CH_3(CH_2)_{16}CH_3$ - BDH Chemicals) was prepared. This was used with and without the addition of 10.0 mg glycopeptide ST208.

For oral administration 0.3 ml of the vaccine emulsion was placed in a gelatin capsule. These capsules were obtained from Polaron Equipment Ltd., Watford, England and are normally used in the embedding process for electron microscopy.

The guinea-pigs were given 5% sodium bicarbonate solution to drink 18 hr prior to oral immunisation, in an attempt to reduce acidity in the stomach. The capsule was held in artery forceps and was placed at the back of the mouth, from where it was instinctively swallowed when the animal's mouth was held closed.

The groups of guinea-pigs received an oral capsule, an i/m injection of 0.5 ml of vaccine, or both. After an interval of two months each animal was given a booster 0.3 ml capsule orally.

Guinea-pigs 321-343 and 410-421. Oral immunisation alone.

These animals received oral capsules containing 0.3 ml of the adjuvant mixture described above, without a mycobacterial component (incomplete adjuvant).

Guinea-pigs 344-364 and 434-445. Oral immunisation alone.

These animals received 0.3 ml oral capsules containing the adjuvant mixture including glycopeptide ST208 (complete adjuvant). Booster doses in incomplete adjuvant were given.

Guinea-pigs 365-385 and 422-433. Primary i/m and oral booster immunisations with incomplete adjuvant

An initial i/m injection of 0.5 ml of the incomplete adjuvant mixture, was followed by oral booster doses of incomplete adjuvant.

Guinea-pigs 386-406 and 446-457

Primary i/m and oral booster immunisations with complete adjuvant

An initial i/m injection of 0.5 ml of the complete adjuvant mixture was followed by oral booster doses of incomplete adjuvant.

(ii) Vaccines Containing Vibrio cholerae organisms

V. cholerae strain NCTC 7254 Inaba was grown for 18 hr at 37°C, in 10 ml of 2.0% protease peptone (Difco) pH 7.5. Aliquots of this culture (0.2 ml) were spread onto the surface of nutrient agar in small petri-dishes, and incubated for 24 hr. The growth from these plates was scraped off, using a sterile wire loop, and resuspended in sterile saline pH 7.0. Samples of this suspension were plated onto T.C.B.S. and nutrient agar to check purity. The vibrio cells were killed by heating the suspension at 55°C for 30 min. The effectiveness of killing was checked by plating onto nutrient agar. The number of vibrio cells in the suspension was determined using a Thoma chamber, and dilutions were prepared in physiological saline, to provide the appropriate concentration of cells for immunisation purposes.

(a) Combined Vaccines of Toxoid and V. cholerae Whole Organisms

Combined vaccines containing both cholera toxoid and V. cholerae organisms were prepared in the incomplete and complete adjuvant mixtures.

Guinea-pigs 458-478. Oral immunisation alone.

These animals received 0.3 ml oral capsules containing incomplete adjuvant mixture for primary and booster immunisations.

Guinea-pigs 500-520. Oral immunisation alone.

These animals received 0.3 ml oral capsules containing complete adjuvant mixture followed by booster doses of incomplete adjuvant.

Guinea-pigs 497-499. Primary i/m and oral booster immunisations.

These animals were injected i/m with 0.5 ml incomplete adjuvant followed by 0.3 ml oral boosters of incomplete adjuvant.

Guinea-pigs 521-541. Primary i/m and oral booster immunisations.

These animals were injected i/m with 0.5 ml complete adjuvant followed by 0.3 ml oral boosters of incomplete adjuvant.

(b) Whole Cell Vaccines

Whole cell vaccines were prepared in a similar manner to the toxoid vaccines. The suspension of V. cholerae in saline was substituted for the toxoid in saline, in both the incomplete and complete adjuvant mixtures.

Guinea-pigs 542-562. Oral immunisation alone.

Oral capsules containing 0.3 ml incomplete adjuvant mixture were used for primary and booster immunisations.

Guinea-pigs 584-604. Oral immunisation alone.

Oral capsules containing 0.3 ml complete adjuvant mixture were used for primary immunisation. Booster doses of incomplete adjuvant were administered.

Guinea-pigs 563-583. Primary i/m and oral booster immunisations.

Injections of 0.5 ml incomplete adjuvant were followed by 0.3 ml oral boosters of incomplete adjuvant.

Guinea-pigs 605-625. Primary i/m and oral booster immunisations.

Injections of 0.5 ml complete adjuvant were followed by 0.3 ml oral boosters of incomplete adjuvant.

10. Collection of Antisera

All animals were exsanguinated by cardiac puncture. Approximately 1.2 ml of blood from each mouse and 20 ml of blood from each

guinea-pig was obtained. The clotted blood was left at 4°C overnight before the serum was removed. Any residual erythrocytes were removed from sera by centrifugation at 2,000 r.p.m. for 10 min. The sera were stored at -20°C until required.

11. Preparation of Gut Tissue Extracts

The small intestine of immunised guinea-pigs was removed at post-mortem. Partially digested food in the intestine was removed and discarded. The intestine was placed in 5.0 ml of cold P.B.S., minced coarsely with scissors, and homogenised using a Silverson blender.

The homogenate was centrifuged at 2,500 r.p.m. for 15 min to deposit the tissue debris. The supernate was removed and was added to an equal volume of 100% saturated ammonium sulphate, $(NH_4)_2SO_4$, solution. The mixture was kept at 4°C for 48 hr and the precipitate was removed by centrifugation at 2,500 r.p.m. for 15 min. The precipitate was extensively dialysed against cold P.B.S. to remove $(NH_4)_2SO_4$. The tissue extract obtained was stored at -20°C.

12. Tests in Animals

A. Ileal loop Test

(i) Toxin Challenge

Guinea-pigs were starved of solid food and provided with a 5% glucose solution for 72 hr, as a preoperative measure. The animals were anaesthetised with ether and sedated with a dose of Veterinary Nembutal (0.6% pentabarbitone sodium; Abbott Laboratories Ltd., Queensborough, Kent) proportional to their weight. A length of small intestine above the caecum, was lifted out of the peritoneal cavity, and the lumen was washed by injection of approximately 10 ml of physiological saline. The contents were easily drained into the caecum.

The intestine was ligated with sterile cotton 2.0 cm and 9.0 cm above the caecum. A third ligature, 7.0 cm above the caecum, was tied loosely, resulting in a 5.0 cm long test loop and a 2.0 cm long interloop. To prevent leakage from the test loops the toxin solution was injected by inserting the hypodermic needle into the 2.0 cm interloop and through the loose ligature into the test loop. The ligature was tightened onto the needle before injection of toxin, and finally tied off after removal of the needle.

Four 5.0 cm loops, separated by 2.0 cm interloops were prepared in each animal. Graded doses of crude cholera toxin (N.I.H.), 1.0 mg, 0.5 mg and 0.25 mg, suspended in 0.5 ml B.G.S., were injected into the three loops respectively, while 0.5 ml B.G.S. was injected into the fourth loop.

The intestine was replaced in the peritoneal cavity and the wound was stitched with monofilament nylon (metric 2; Ref. No. V4022; Arnolds Veterinary Products Ltd., Ayr) and the animal was allowed to recover from the anaesthetic. After 8 hr the animal was sacrificed and the ileal loops were removed. Measurements of the length and diameter of the loops were made using calipers (Camlab, Cambridge, type 6921). The accumulated fluid in each loop was emptied into a small beaker and the volume was measured in a pipette.

(ii) Viable *V. cholerae* Challenge

A 0.5 ml aliquot of an overnight culture of *V. cholerae* NCTC 7254, grown in 2% protease peptone pH 7.5, was transferred to 20 ml of this medium in a 250 ml conical flask. The culture was incubated, without shaking, at 37°C for 4 hr. The viable cell count of the culture was estimated by the method of Miles and Misra (1938). The

range of repetitive viable cell counts of V. cholerae was between 1.6 and 2.1 x 10^7 cells/ml. Basu and Picket (1969) found 1 x 10^6 V. cholerae cells to be sufficient to set up an effective ileal loop challenge; 0.1 ml of a 4 hr culture was therefore used for ileal loop testing.

Ileal loops were prepared in guinea-pigs as described above. Two interloops of approximately 2.0 cm were left between the test loop, inoculated with V. cholerae, and the control loop inoculated with 0.1 ml sterile 2% proteose peptone.

The animals were sacrificed 16 hr after challenge and the ileal loops were examined. Measurements of length and diameter of the loops were made. To avoid dissemination of V. cholerae, instead of measuring the fluid volume contained in the loop, the whole loop was cut out of the intestine, immersed in disinfectant solution, and the displaced volume was measured.

B. Intradermal Blueing Tests in Rabbits

(i) Toxin Titration

Serial two-fold dilutions of crude cholera toxin (N.I.H.) were prepared in B.G.S. These dilutions (0.1 ml) were injected intradermally (i/d) into the skin of rabbits, previously shaved with Oster, Animal Grooming Clippers (Model A-5). After 18-24 hr, 3 ml of a 5.0% solution of Pontamine Sky Blue 6XB (Difco) were injected i/v into the ear. One hour later the diameters of the zones of blueing on the skin were measured. The toxin dose which produced a blue zone of 7 mm in diameter was equivalent to one blueing dose (1 B.D.).

(ii) Inhibition of Intradermal Blueing

Serial two-fold dilutions of each antiserum, in 0.2 ml amounts,

were prepared in B.G.S. Crude cholera toxin (N.I.H.) solution (120 B.D./0.2 ml) was added to each dilution and the mixtures were incubated at 37°C for 1 hr, with intermittent shaking. As soon as possible after incubation each mixture (0.1 ml) was injected i/d into the shaven skin of a rabbit.

Thereafter the technique was as described above. A standard titration of the crude toxin preparation was carried out simultaneously in each animal. The end-point of the antitoxin titration was recorded as that dilution which yielded an area of blueing 4 mm in diameter.

C. Intradermal Blueing Test in Mice

Dilutions of crude cholera toxin (N.I.H.) were prepared as for the rabbit intradermal blueing test. Each dilution (0.1 ml) was injected into four sites in the skin of hairless mice. After 24 hr, 0.2 ml of a 1% solution of Pontamine Sky Blue dye was injected i/v into the tail. The mouse skins were removed 3 hr after the administration of the dye, and the outer and inner surfaces were examined for blueing reactions.

13. Passive Haemagglutination (P.H.A.) Test

Cholera toxin, CTII (10 ml) (see p. 88) was added to 90 ml P.B.S. pH 7.2. Washed, packed sheep erythrocytes (4.0 ml) were added followed by 2.5% aqueous glutaraldehyde solution, with continuous stirring. The mixture was stirred at room temperature for 1 hr to allow coating of the erythrocytes with the toxin. The toxin-coated cells were deposited by centrifugation at 2,000 r.p.m. for 10 min, the supernate was discarded and the pellet of cells was washed three times in P.B.S. pH 7.2. The coated erythrocytes were finally resuspended in 1.0% normal rabbit serum in P.B.S. and stored at -20°C.

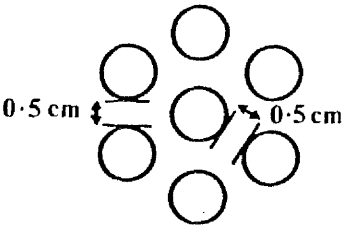
Control cells were prepared by the same procedure with the omission of cholera toxin.

Before use, a 1.0% suspension of coated erythrocytes was prepared. A capillary tube was filled with the erythrocyte suspension, sealed at one end and spun at 1,000 rpm for 10 min in an Haematocrit centrifuge, M.S.E. The packed cell volume in the capillary tube was read using an haematocrit and the suspension was diluted accordingly in P.B.S. containing 1.0% normal rabbit serum.

Serial two-fold dilutions of each antiserum were prepared in P.B.S. pH 7.2, in 0.025 ml volumes in a Cooke Microtitre plate (Sever, 1962), from N →1/512, using Titertek automated microtitration equipment (Flow Laboratories, Irvine). Toxin-coated cells (0.025 ml of the 1.0% suspension) were added to each well on one plate, and 0.025 ml of the 1.0% control-cell suspension was added to each well of a duplicate plate. The contents of each well were mixed by gently tapping the sides of the plates. The plates were covered, to prevent evaporation during incubation at 37°C for 1 hr and storage overnight at 4°C. The end-point of this titration was taken as the highest dilution of antiserum showing visible haemagglutination. Where non-specific agglutination occurred with the control suspension, the titre obtained was subtracted from the end-point with the toxin-coated cells.

14. Ouchterlony Double Diffusion in the Gel Precipitin Test

Molten gel diffusion medium (20.0 ml) was poured into scratch-free glass petri dishes. The agar was hardened by storing the plates at 4°C for 1 hr before cutting out the template pattern described by King (1957):—



One drop of molten medium was added to each well to prevent leakage of reagents underneath the agar layer. Antigen and antibody-containing solutions were used to fill opposing wells and the resulting precipitin lines were examined after incubation of the plates at 37°C for 24 hr.

15. V. cholerae Agglutination Test

A. Microtitration Assay

A suspension of V. cholerae NCTC 7254 was prepared as described for whole cell vaccine production (p. 94). The optical density of this suspension was adjusted visually using a W.H.O. International Reference Preparation of Opacity, as described by Perkins et al (1973). The reference preparation is a perspex rod of 10 International Units (I.U.) of opacity, which is placed in a 6 x 5/8 glass tube for use. The cell suspension was diluted in identical glass tubes and the dilutions were compared to the standard by simultaneously looking through reference and dilution at bold black type. The dilution which had the same visual opacity as the standard contained 10 I.U./ml; 1 I.U. is approximately equivalent to 1 x 10^8 cells.

Serial two-fold dilutions of serum were prepared in microtitre plates (N → 1/128), using the Titertek equipment, in 0.025 ml volumes. V. cholerae cells (0.025 ml of a 1.0 I.U. suspension) were added to

each well and the sides of the plate were tapped gently to ensure mixing. The plates were covered and incubated at 37°C for 1 hr.

Plates were kept at 4°C overnight before examination. The last serum dilution in which no button of unagglutinated vibrios was present, was recorded as the end-point.

B. "H" Suspension

An overnight culture of V. cholerae NCTC 7254, grown in 2.0% proteose peptone, was centrifuged at 6,000 r.p.m. for 30 min. The pelleted vibrios were resuspended in 20.0 ml of saline containing 0.1% formalin (v/v) and kept at room temperature for 1 hr.

This suspension was diluted in formol saline to an optical density of 2.0 I.U. before 0.5 ml aliquots were added to 0.5 ml serial dilutions of test antisera in 4 x ½" test tubes. These mixtures were transferred to Dreyer tubes, as was a control suspension containing no antiserum. The mixtures were incubated at 56°C in a water-bath for 2 hr before examination for agglutination. After storage at 4°C pellets of agglutinated vibrios accumulated at the bottom of the tubes. The degree of pelleting was recorded.

C. "O" Suspension

V. cholerae NCTC 7254 was grown as described above. The culture was heated at 55°C for 30 min in a water-bath, to kill the organisms, before the centrifugation step. The vibrios were resuspended in absolute alcohol and placed in a 45°C water-bath for 10 min. The suspension was centrifuged at 2,500 r.p.m. for 30 min, the supernate was removed and the vibrios were washed and resuspended in sterile saline. The optical density was adjusted and the agglutination test was carried out as described above.

16. Complement Fixation Test

The complement contained in sera to be tested was inactivated by incubation at 56°C for 30 min. Serial doubling dilutions of each serum were prepared in 0.025 ml volumes in V.B.S. across the 12-well rows of a microtitre agglutination plate, N - 1/2048. A suspension of heat-killed V. cholerae (10^9 cells/ml, 0.025 ml) was added to each well. Guinea-pig complement (Wellcome Reagents Ltd., Beckenham, England, 2 units/ml) was added to each well in 0.025 ml amounts, and the reagents were thoroughly mixed by gently tapping the sides of the plate. Several controls were set up simultaneously; (a) normal serum was substituted for test serum, (b) V.B.S. was substituted for serum, and (c) V.B.S. was substituted for V. cholerae suspension.

After incubation at 37°C for 40 min, 0.025 ml of sensitised sheep erythrocytes, prepared by adding an equal volume of 2% erythrocytes to haemolysin solution (Wellcome Reagents Ltd., 2 haemolytic units (H.U.) per 0.5 ml), was added to each well. The contents of the wells were mixed and the tests reincubated at 37°C. During the 1 hr incubation period the erythrocytes were maintained in suspension by tapping the side of the plate at regular intervals.

After storage at 4°C for 18 hr, the end-point, i.e. the dilution of serum preceding that which allowed 100% lysis of the sensitised erythrocytes, was determined.

RESULTS

1. Gel Filtration of Enterotoxin

Several 10 g samples of crude cholera toxin (NIH) were prepared for gel filtration (see p. 89). Three methods of enterotoxin purification were compared:

A. Filtration through Sephadex G-100 followed by Sephadex G-200

Crude enterotoxin, partially purified by $(NH_4)_2SO_4$ precipitation, gave the elution profile shown in Fig 5 on a Sephadex G-100 column of 25 ml void volume (v.v.). Since the exclusion limit of this gel is around 100k daltons, the cholera enterotoxin (84k daltons) was expected to be eluted soon after the void volume. Fractions contained in the first peak (1), were pooled and concentrated by $(NH_4)_2SO_4$ precipitation at $4^{o}C$ for 18 hr. The mixture was centrifuged at 8,000 rpm for 30 min and the pellet was resuspended in B.G.S. The solution was dialysed against 2 litres of B.G.S. at $4^{o}C$ for 24 hr.

The dialysate was applied to a column of Sephadex G-200, 24 ml v.v., and eluted with B.G.S. (Fig 6). The four 2 ml fractions of the single peak were examined in Ouchterlony gel diffusion against antiserum from guinea pig DSTGP 298, which was known to give precipitin lines with the immunopurified toxoid preparation. Faint precipitin lines were observed between the guinea-pig serum and column fractions. These fractions were also injected, in 0.1 ml volumes, into the skin of a hairless, hrhr, mouse, to determine their blueing activity; blue zones of approximately 20 mm were produced, on the inner surface of the skin, by each fraction.

The four column fractions were pooled and concentrated, to a volume of 4 ml, using Carbowax (polyethylene glycol), before testing for activity in the rabbit intradermal blueing test.

Figure 5. Elution of Cholera Enterotoxin from Sephadex G-100

(void volume = 25 ml, flow rate = 10 drops/min)

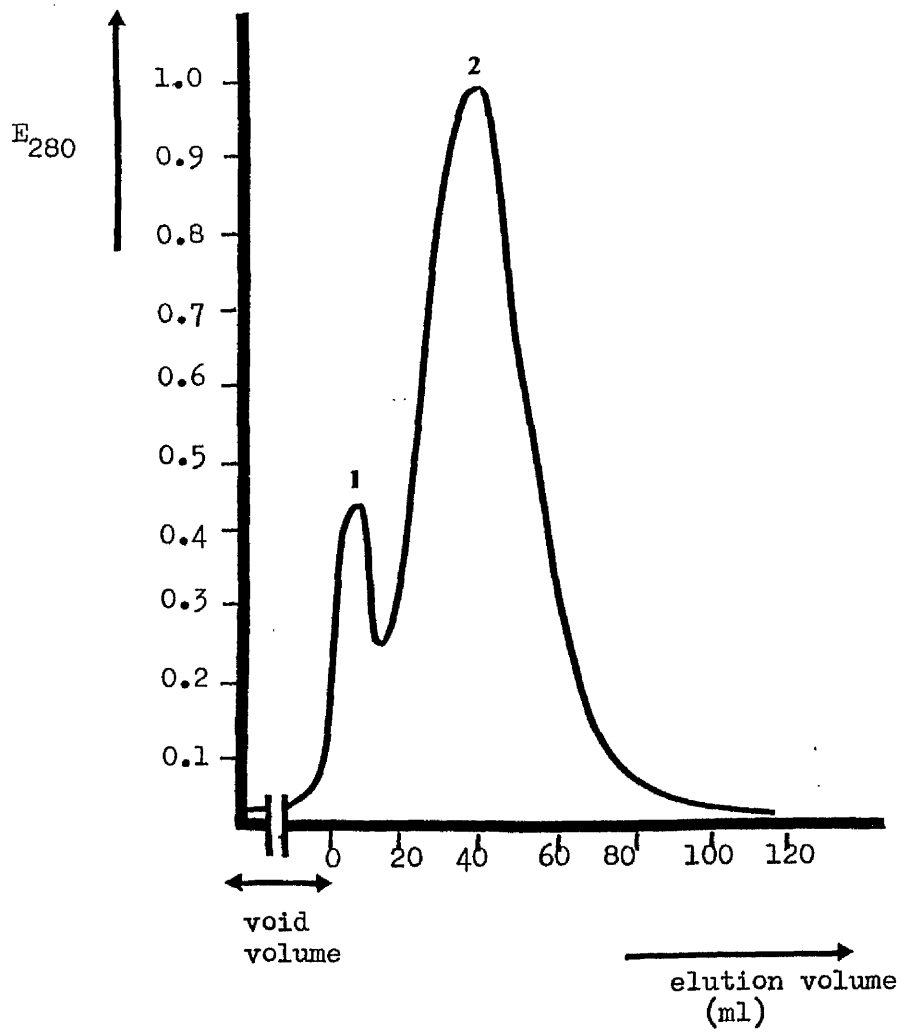
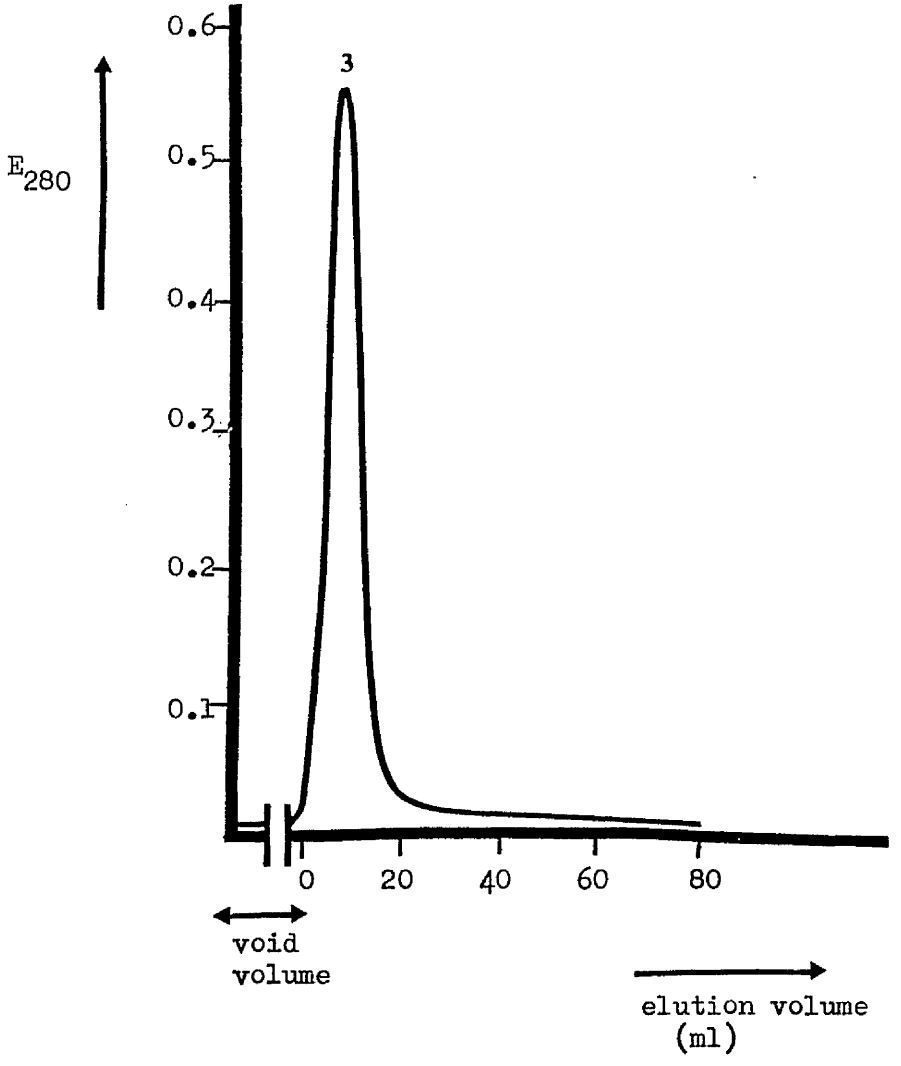


Figure 6. Elution of Peak (1) fractions (Fig 5) from Sephadex G-200

(void volume = 24 ml, flow rate = 10 drops/min)



B. Filtration through Sephadex G-100 and G-200 columns in series

The two Sephadex columns were connected in series and the $(NH_4)_2SO_4$ precipitate of 10 g of crude NIH enterotoxin was pipetted onto the bed of Sephadex G-100. Elution was carried out using BGS. The material in peak (2) (Fig 5) was retained, in the G-100 column, while the enterotoxin-containing peak was eluted and filtered again on the G-200 column. The elution profile is shown in Fig 7. The presence of active enterotoxin in the main peak, (4), was demonstrated by the mouse intradermal blueing test. This purified material contained 1 B.D. per 2 μg of protein, in comparison to the crude NIH enterotoxin, which contained 1 B.D. per 4 μg of protein.

C. Filtration through a Sephadex G-200 column

Elution of partially purified NIH enterotoxin from a Sephadex G-200 column, (60 ml void vol) showed an isolated enterotoxin containing peak, (6) (Fig 8). The presence of active enterotoxin was confirmed by mouse intradermal blueing.

This method was judged to be as efficient as the other two methods in separating a peak containing partially purified enterotoxin from a brown pigment and low molecular weight substances in the culture filtrate $(NH_4)_2SO_4$ precipitate.

D. Purification of enterotoxin obtained from V. cholerae NCTC 7254 Inaba serotype and comparison with the elution properties of NIH toxin

Cholera enterotoxin was produced from V. cholerae NCTC 7254 (see p. 85). The elution profile obtained when this material was applied to a Sephadex G-200 column, was compared to the elution profile

Figure 7 Elution of Cholera Enterotoxin from Sephadex G-100 Connected in Series to Sephadex G-200

(total void volume = 49 ml, flow rate = 10 drops/min)

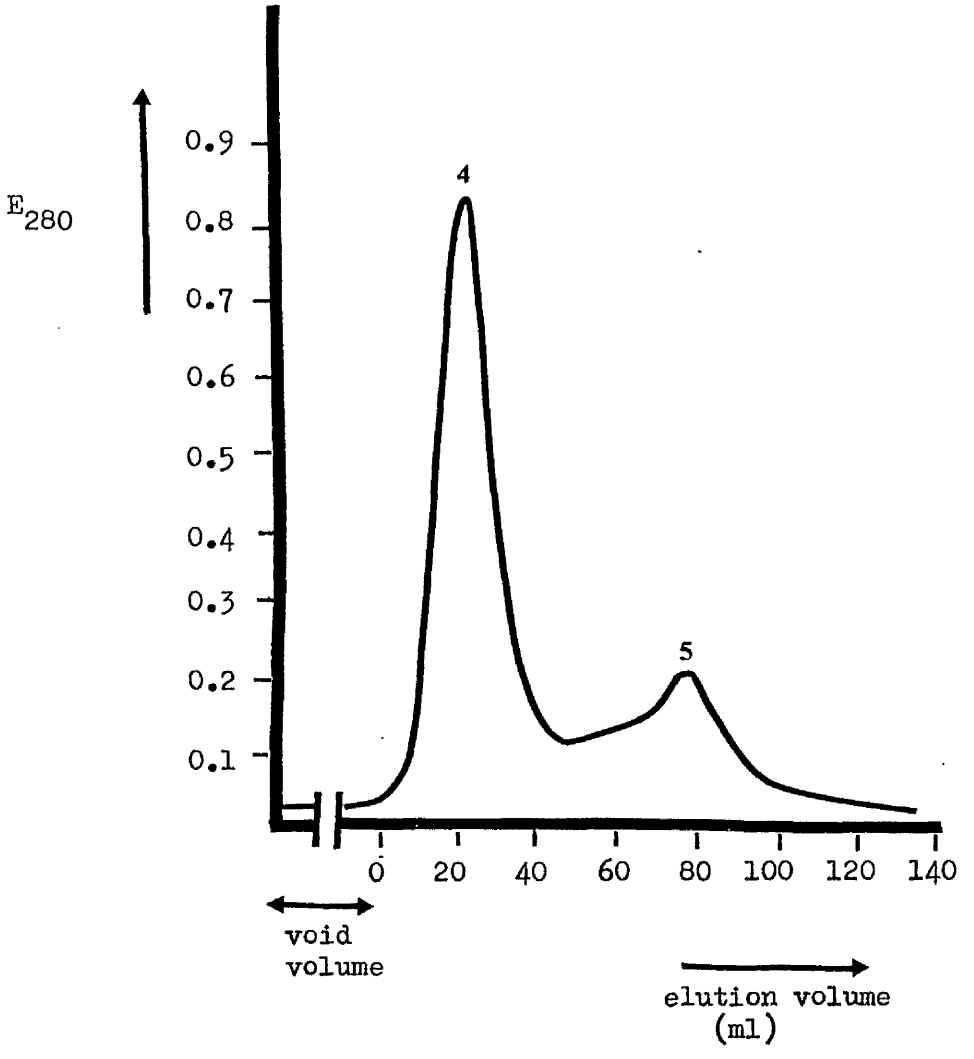


Figure 8. Elution of cholera enterotoxin from Sephadex G-200 (void volume = 60 ml, flow rate = 10 drops/min).

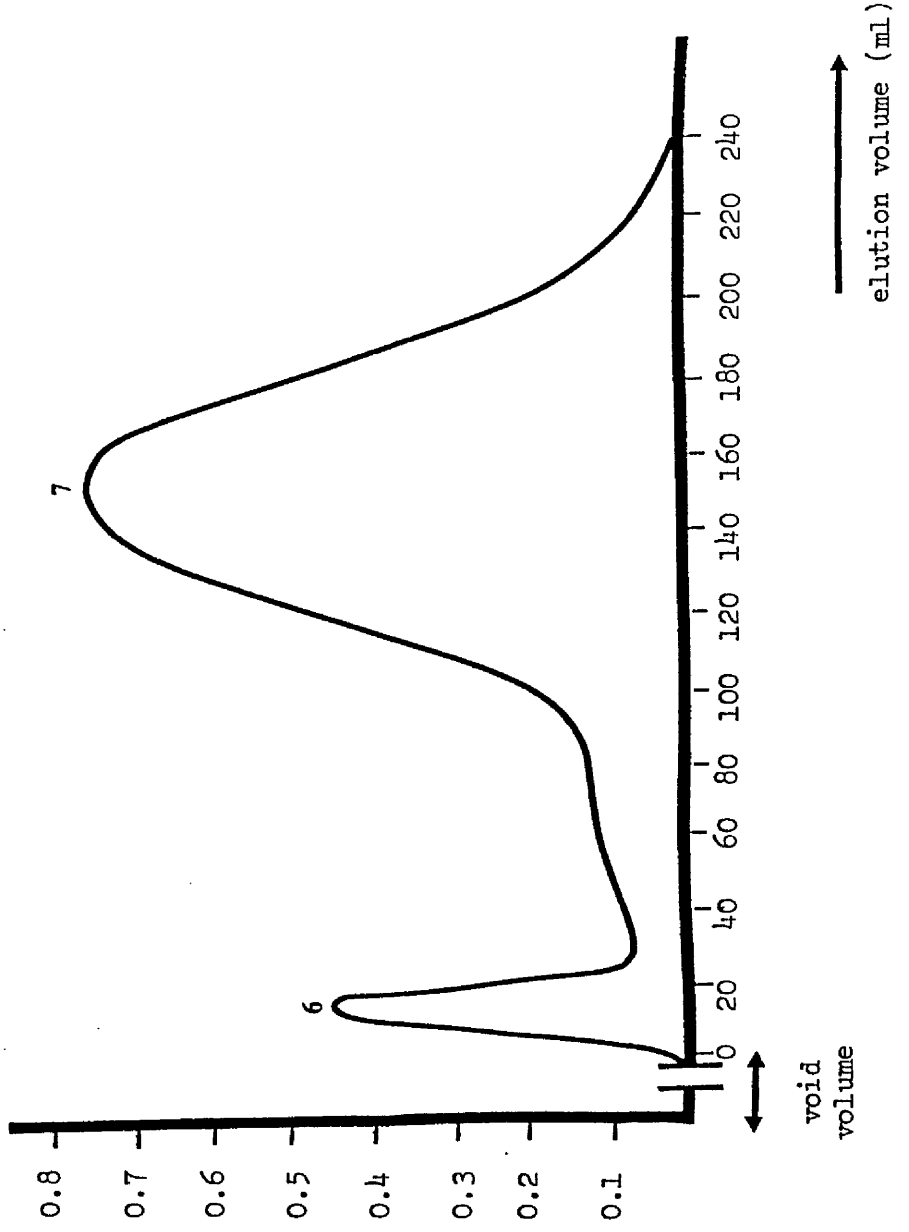
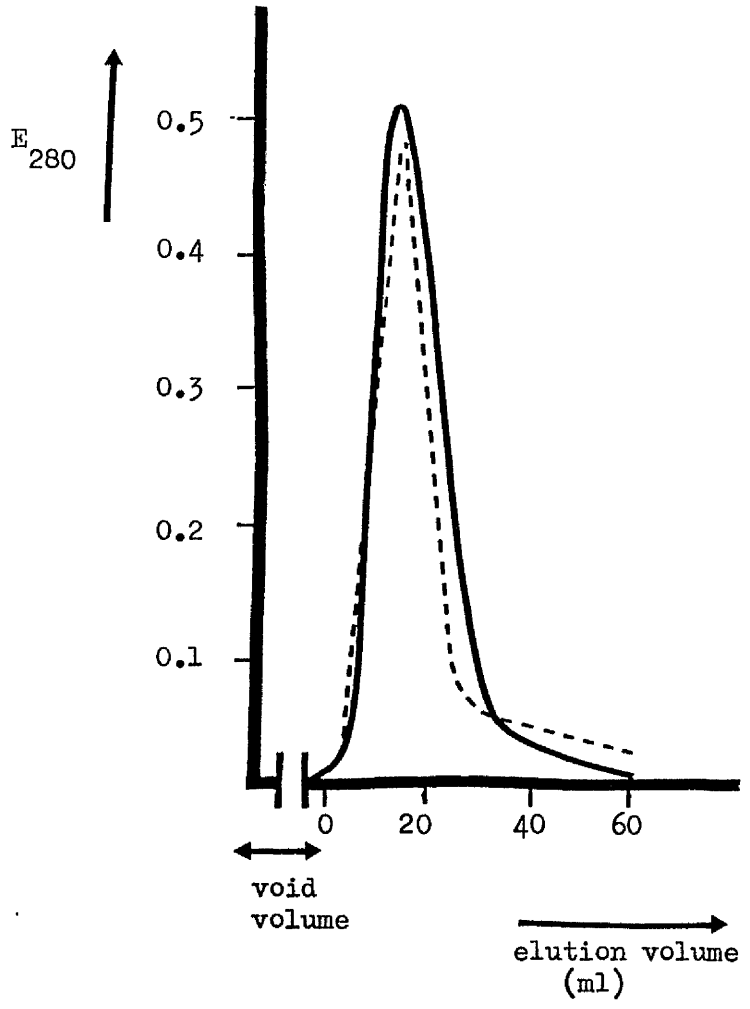


Figure 9 Elution of Partially Purified Cholera Enterotoxin from Sephadex G-200

(void volume = 60 ml, flow rate = 10 drops/min)



——————— Pooled fractions from Peak (6) (Fig 8)

- - - - - - - - - - - V. cholerae strain NCTC 7254 enterotoxin purified as NIH enterotoxin (Fig 8)

obtained when partially purified NIH enterotoxin was eluted from the same column (Fig 9). The enterotoxic activity of the preparation from V. cholerae NCTC 7254 was confirmed by intradermal blueing in mice.

2. Response in Rabbits to Intradermal Injection of NIH Toxin

The results of 22 replicate titrations of crude cholera toxin (NIH) were averaged. The $\log_{10}$ of the toxin dose was plotted against the average blue zone diameters (Fig 10). As shown, 4 μg of this toxin was equivalent to 1 B.D.

A typical response obtained on titration of cholera toxin in the rabbit intradermal blueing test is shown in Plate 6.

3. Response in Rabbits to Intradermal Injection of Wellcome Toxoid

The toxoid preparation used in vaccination schedules was examined for active toxin by the rabbit i/d blueing test. Serial two-fold dilutions, in B.G.S., of the preparation were injected in 0.1 ml volumes into the skin. The diameter of the blue zones measured after injection of the blue dye were:-

Toxoid dilution	1/2	1/4	1/8	1/16	1/32
diameter of blue zone (mm)	8.2	6.9	5.3	4.5	-

It was apparent that 0.1 ml of the 1/4 dilution of toxoid contained 1 B.D. ($\sim$7 mm zone). Thus the preparation contained approximately 40 B.D. per ml or 20 B.D. per mg of protein.

4. Response in Mice to Intradermal Injection of NIH Toxin

Graded doses of NIH cholera toxin were injected i/d into hairless mice. The 4 doses of toxin studied were interchanged among

Figure 10. Blueing response in rabbits to intradermal injection of N.I.H. toxin

($\pm$ S.E.M. of 25 replicate titrations)

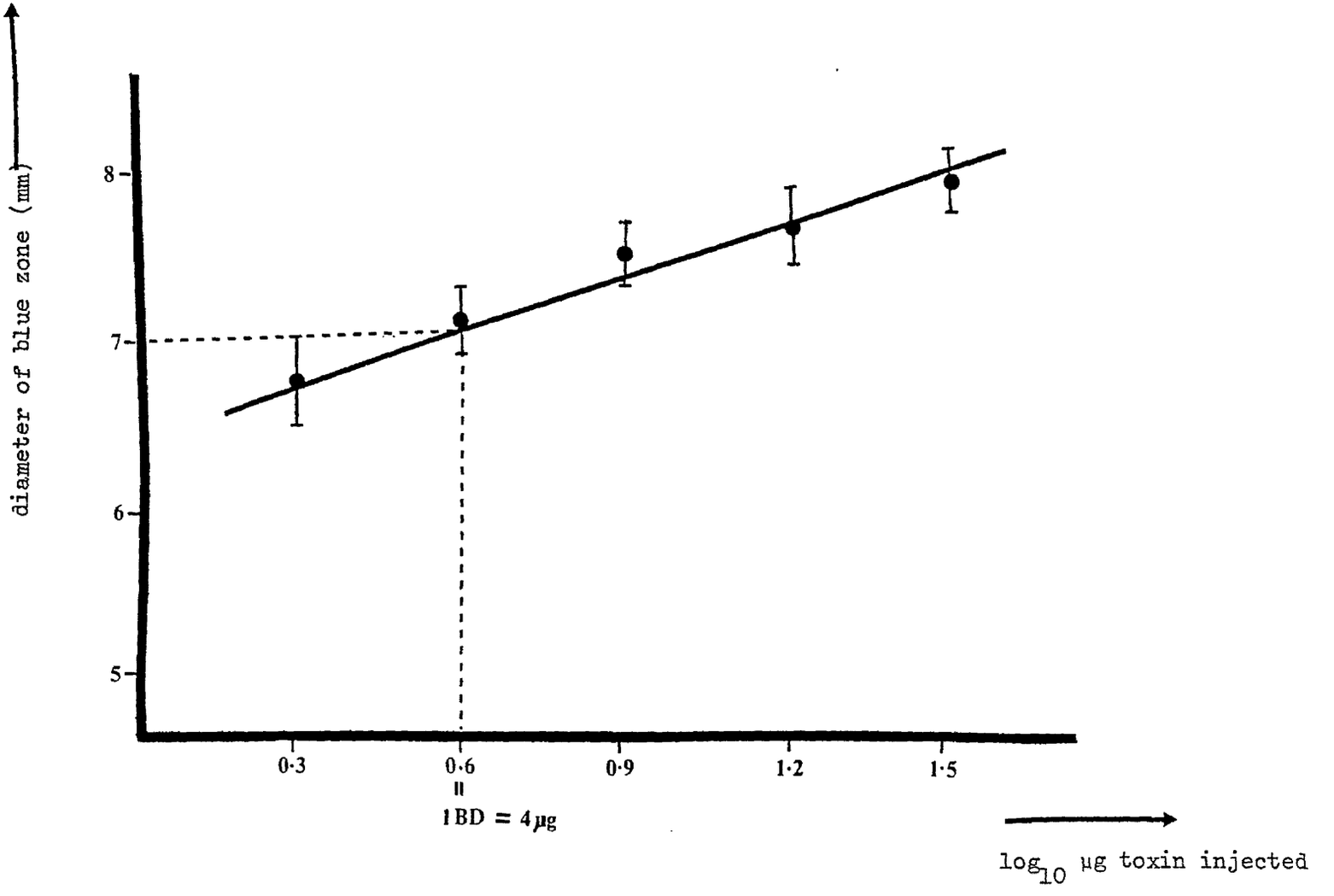
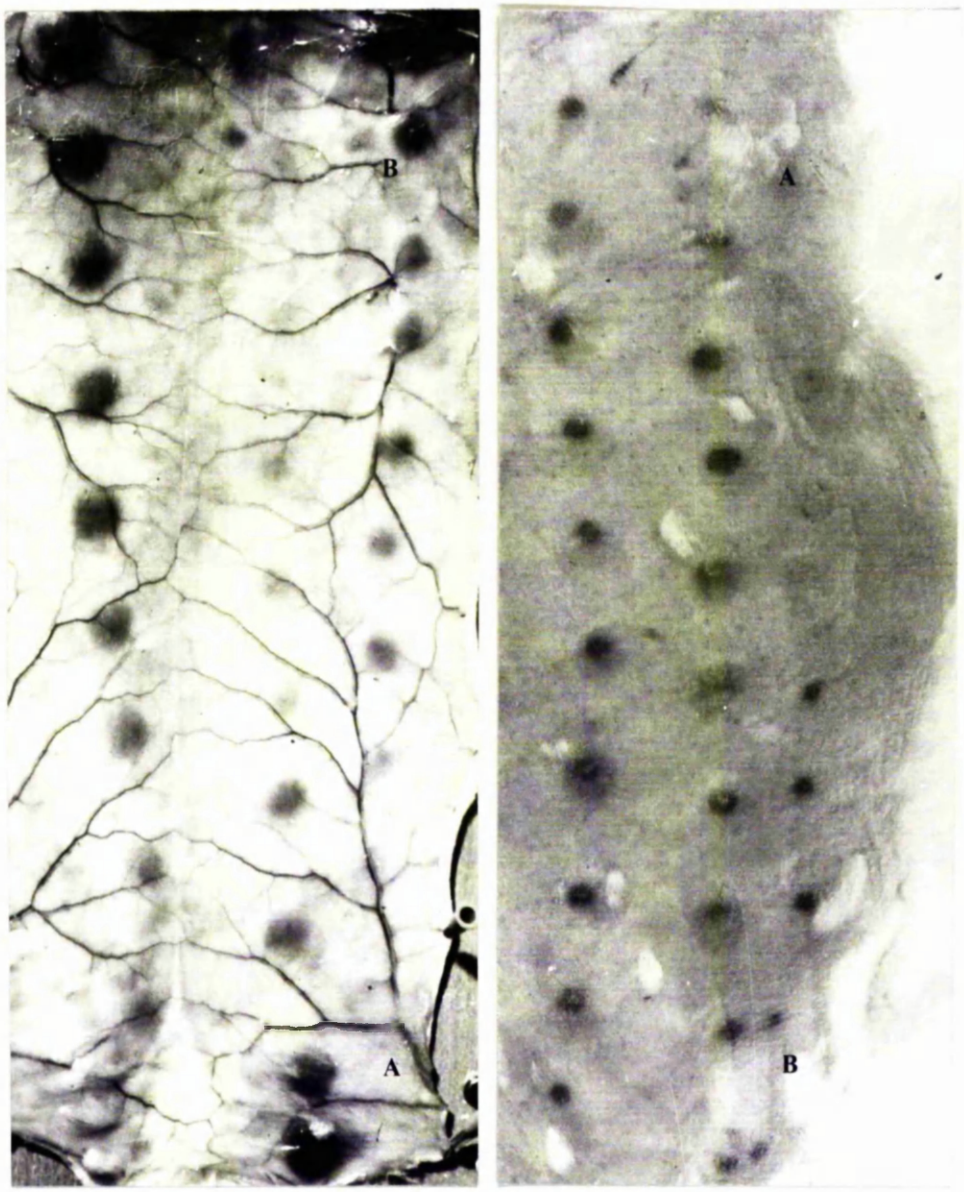


Plate 6. Intradermal titration of cholera toxin as seen on the outer and inner surfaces of rabbit skin.

Inner Surface	Outer Surface

Note the decreasing blueing reaction from the lowest dilution (A) to the highest dilution (B).


B
A
A
B

the 4 available injection sites on each mouse. The results were read 3 hr after i/v injection of Pontamine dye (Table 2).

The blueing reaction on the outer surface of the mouse skin was rather faint and difficult to read. One hr after dye injection there were blue zones, 4-6 mm in diameter, on the outer surface of the skins. After 3 hr the colour was slightly more dense and the zones were measured at this time. There was no visible firm swelling on the outer surface of the skin, as there was with the rabbit skin test.

Mice were sacrificed and their skins were carefully removed 3 hr after dye injection. On the inner surface of the skin swollen blue zones were clearly visible at the sites of injection and were more easily measured (Plate 7). The zones were not always circular, however, and this decreased the accuracy of the test; the mean of two measurements for each spot are given in Table 2.

5. Enterotoxin Titration in Guinea-pig Ileal Loops

A typical ileal loop test with NIH enterotoxin is shown in Plate 8.

Fluid accumulation (ml/cm) in ileal loops increased sharply from base-line levels, on injection of graded doses of NIH enterotoxin, between the 200 μg and 250 μg dose levels (Fig 11). The data from a number of titrations is contained in Appendix 1.

In toxin neutralisation experiments in vivo it was decided to use challenge doses of 250 μg, 500 μg and 1000 μg of enterotoxin.

6. Immunisation Studies in Mice

Immunisation studies in mice had four main aspects, (i) choice of best available immunogen for antitoxin production; (ii) comparison

Table 2. Intradermal blueing test in mice

µg NIH toxin injected i/d	mm diameter of blue zone measured on skin	
	inner surface	outer surface
2.0	7.0, 6.0, 5.0, 6.0	* 5.0, 4.5, 5.8
4.0	4.5, 8.1, 8.0, 6.5	* 4.0, 4.5, 6.5
8.0	8.0, 7.5, 8.0, 8.5	* - . 6.5, 7.5
16.0	10.0, 10.5, 10.0, 8.5	* 4.5, 6.0, 7.5

*The areas of blueing were not measured externally, due to the dye leaking into the skin from scratches caused by fighting during the 24 hr test period.

Plate 7. Blueing response to intradermal injection of cholera toxin into mice, as seen on the inner surface of the skin.

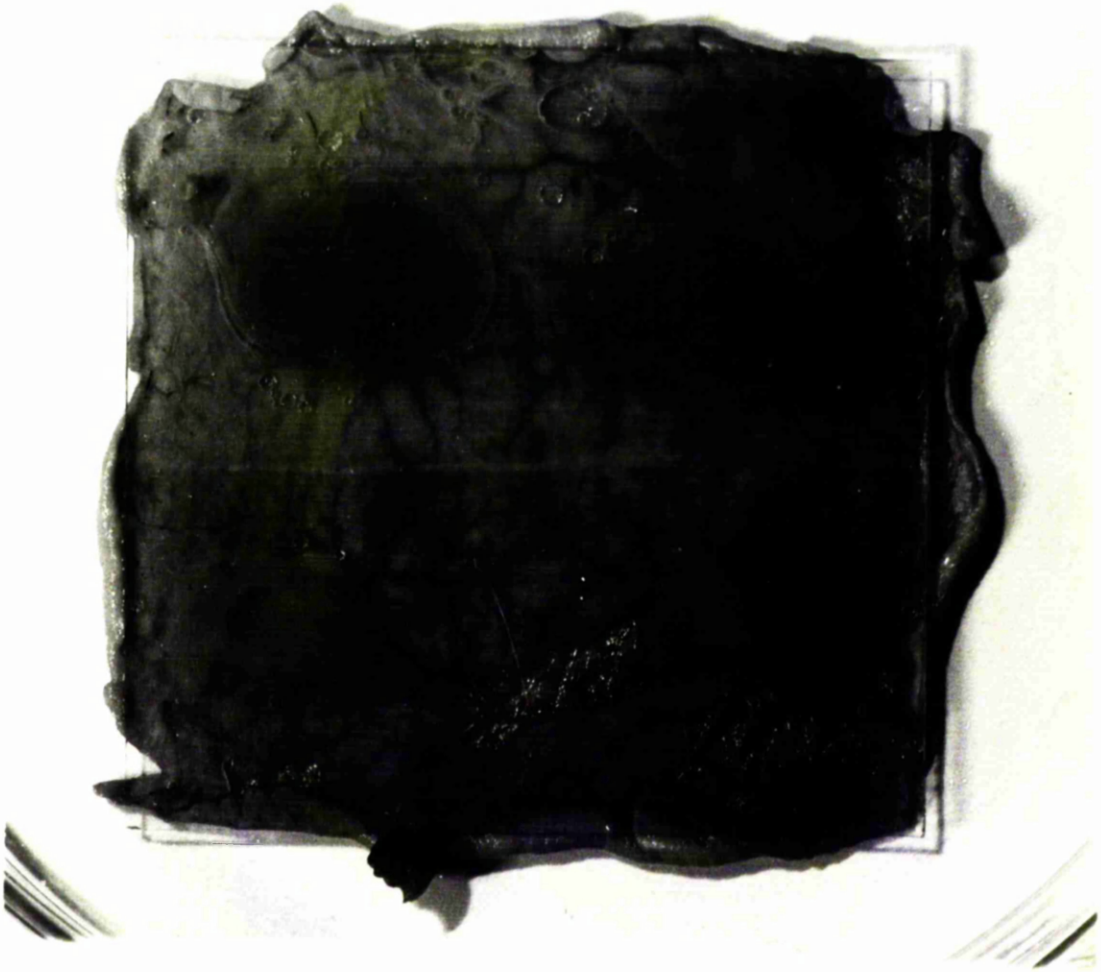


Plate 8. Fluid accumulation in guinea-pig ileal loops eight hours after injection of graded doses of N.I.H. toxin.

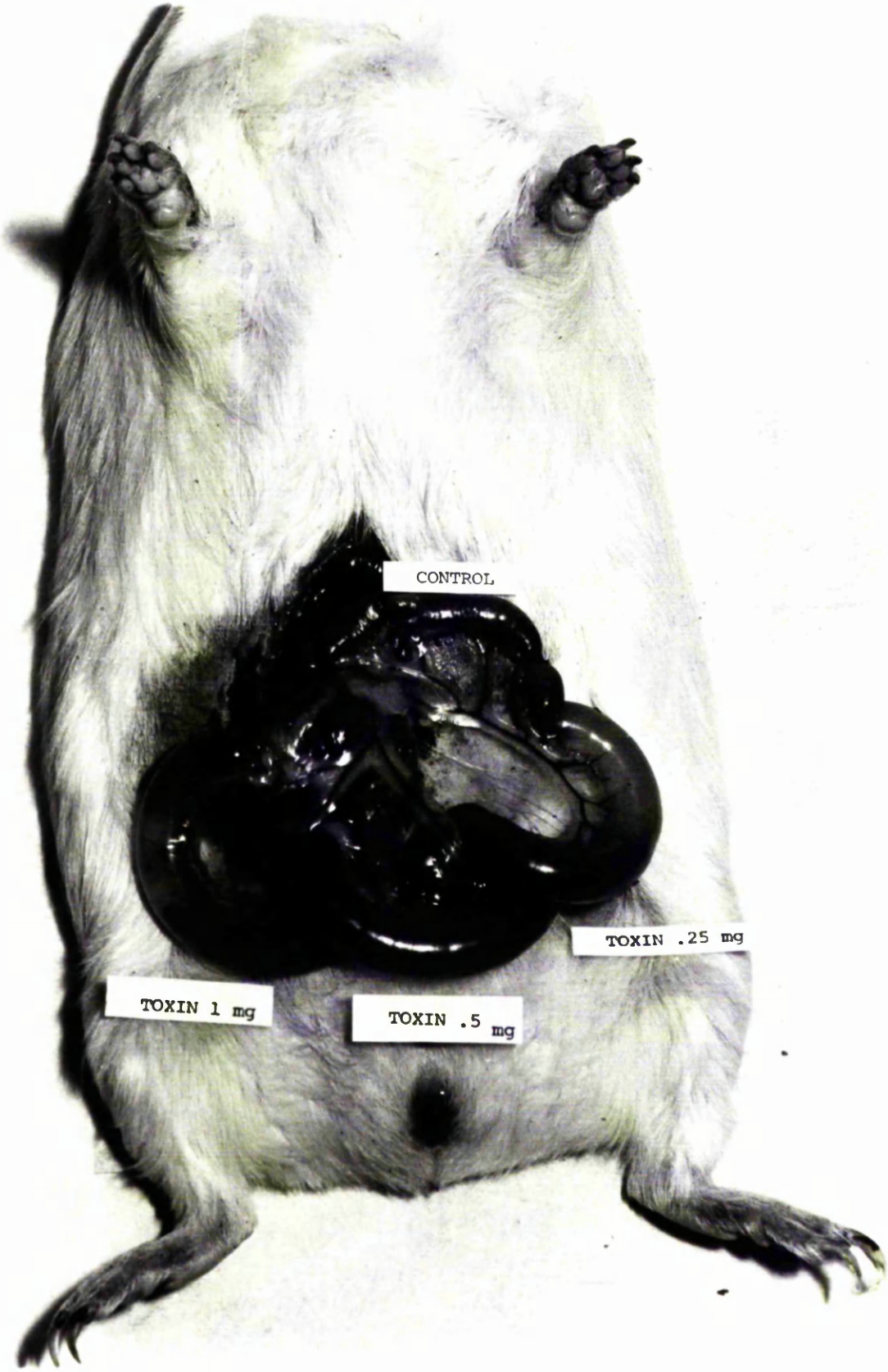
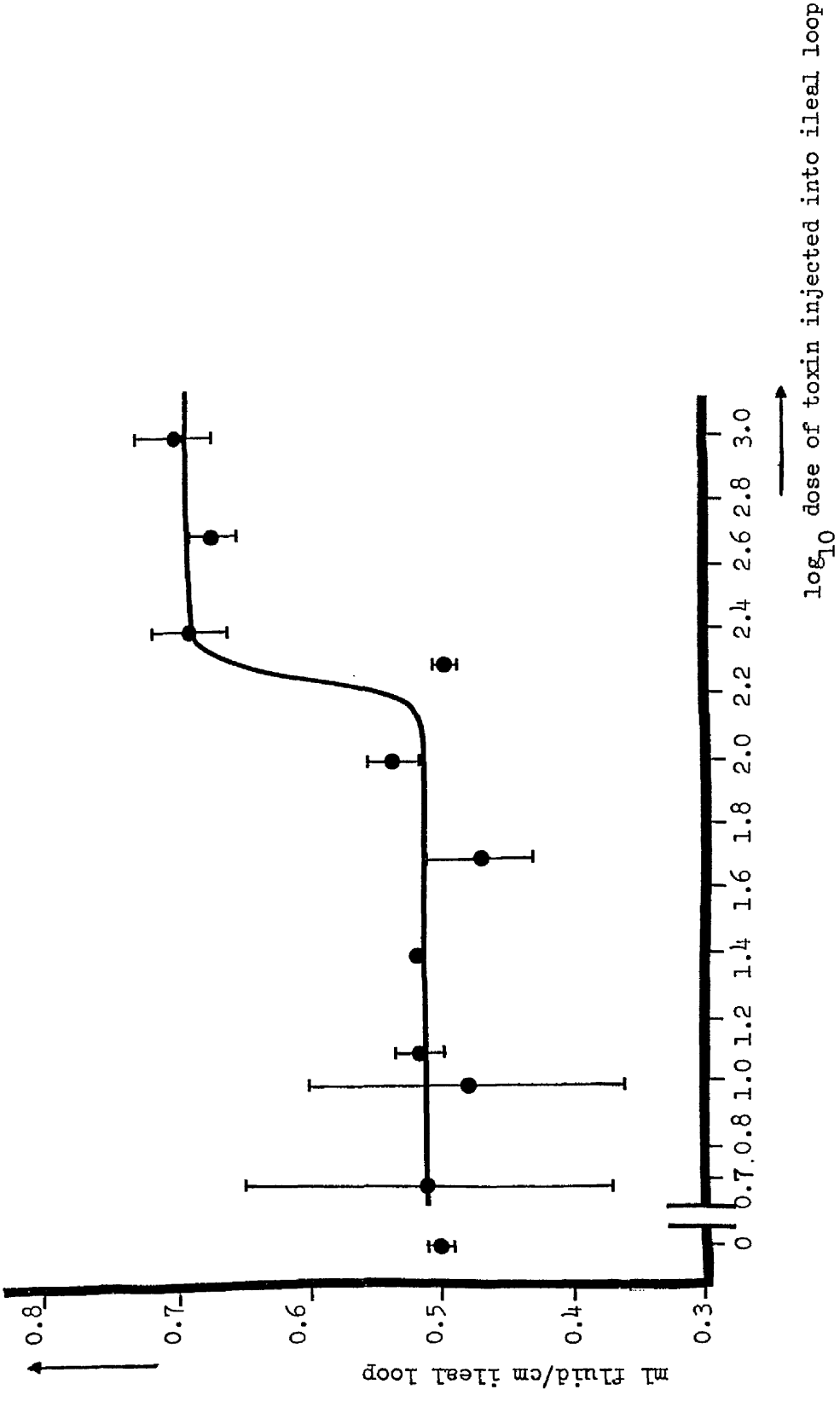

CONTROL
TOXIN .25 mg
TOXIN 1 mg
TOXIN .5 mg

Figure 11. Fluid Accumulation per cm of Ileal Loops Injected with NIH Toxin



of varied toxoid doses incorporated into different vaccine mixtures; (iii) effect of inclusion of V. cholerae organisms into toxoid vaccines; (iv) possibility of stimulating an antibacterial antibody response by injection of whole dead V. cholerae organisms. Each of these aspects will be considered in turn.

A. Comparison of NIH Toxin and Immunopurified Toxoid in Vaccines

An initial experiment was done in mice to determine the relative benefits of using either crude enterotoxin or purified toxoid for immunisation. Mice were given a single injection (0.1 ml, i/p) containing the toxin or toxoid preparation in saline, incomplete adjuvant or complete adjuvant with mycobacterial glycopeptide ST208. After 21 days the antitoxin levels in the serum were measured.

It was apparent that the antitoxin levels stimulated by the crude enterotoxin were low in all animals. The animals injected with toxoid in incomplete and complete adjuvant mixtures showed significantly greater levels of antitoxin than found in the animals which received toxoid in saline (Table 3). The incorporation of ST208 into the injection mixture did not stimulate a further increase in the antitoxin levels.

B. Intraperitoneal Administration of Toxoid in various Injection Mixtures

Twenty one days after a single i/p injection of variable amounts of toxoid in saline, incomplete adjuvant or complete adjuvant containing varying quantities of M. tuberculosis strain DT organisms or mycobacterial glycopeptide, ST217, mice were sacrificed and blood was collected. Antitoxin levels were measured using the intradermal blueing inhibition test (Plate 9) and the haemagglutination of cholera toxin-coated-erythrocytes.

Table 3. Serum antitoxin response in mice to immunisation with either crude NIH cholera toxin or Wellcome immunopurified toxoid

Injection	µg Cholera toxin (NIH)/mouse 160		80		µg Wellcome toxoid/mouse 80		40	
Medium	AU	PHA	AU	PHA	AU	PHA	AU	PHA
saline	1.2	4	1.2	2	9.6	128	9.6	256
incomplete adjuvant	1.2	2	1.2	1	76.8	256	38.4	256
complete adjuvant containing 100 µg ST208/mouse	1.2	1	2.4	1	38.4	1024	38.4	128

AU = antitoxin units/ml serum)
) serum pooled from 5 mice
PHA = reciprocal titre/0.025 ml serum)

Plate 9. Neutralisation of intradermal blueing activity of cholera toxin by antiserum.

Un-neutralised

Neutralised

With toxoid in saline or incomplete adjuvant there was a general increase in antitoxin levels according to the dose of toxoid in the vaccine (Table 4). The incorporation of 500 μg M. tuberculosis organisms into the vaccines did not produce a greater response than the controls. When the quantity of organisms was reduced to 60 μg, 30 μg or 15 μg, stimulated levels of antitoxin were detected by the i/d blueing test with the 40 μg, 20 μg and 10 μg doses of toxoid. With 10 μg, 5 μg or 2.5 μg amounts of M. tuberculosis a stimulatory effect was observed only with the two higher doses of toxoid (Table 5). The PHA titres appeared to be more variable and although some values were greater than those in control animals, in general there was little evidence of increased titres of haemagglutinating antibodies (Table 6).

Incorporation of the mycobacterial glycopeptide ST217 into toxoid vaccines significantly increased antitoxin levels as measured by the i/d blueing test (Table 7). The antitoxin response was greater than that observed with any dose of toxoid in saline, incomplete adjuvant or complete adjuvant containing similar weights of M. tuberculosis organisms. The effect was most pronounced with 60 μg, 30 μg and 15 μg of ST217, where the immune response was markedly stimulated at all doses of toxoid. With 10 μg, 5 μg or 2.5 μg of ST217 the stimulatory effect was noted with the vaccines containing 10-40 μg toxoid. Some increased PHA titres were evident with vaccines containing 30 μg and 15 μg ST217 (Table 8) but with other vaccines there did not appear to be increased titres of haemagglutinating antibody.

Fig 12 shows a graphical representation of the geometric mean antitoxin response (as A.U./ml) obtained in groups of mice on immunisation with the various vaccine mixtures used.

Table 4. Antitoxin Response to Injection of Wellcome Toxoid in Saline and Incomplete Adjuvant

Injection Medium	µg Wellcome Toxoid per mouse					
	40	20	10	5	2.5	1.25
	Passive haemagglutination (reciprocal of titres/0.025 ml serum)					
saline	256	512	32	8	2	4
	512	64	4	8	2	2
	512	16	16	4	2	8
	2048	16	32	1	2	-
incomplete adjuvant	512	256	128	2	2	1
	512	16	256	4	32	2
	512	32	64	2	64	-
	16	4	32	2	2	1
	Antitoxin units/ml serum					
saline	76.8	19.2	9.6	9.6	2.4	4.8
	19.2	9.6	9.6	4.8	2.4	2.4
	19.2	9.6	9.6	4.8	1.2	1.2
	19.2	9.6	9.6	4.8	1.2	1.2
incomplete adjuvant	19.2	9.6	9.6	9.6	4.8	< 0.6
	19.2	4.8	9.6	2.4	9.6	< 0.6
	19.2	4.8	9.6	1.2	4.8	< 0.6
	19.2	4.8	9.6	0.6	1.2	< 0.6

Table 5. Antitoxin Response to Injection of Toxoid in Incomplete Adjuvant containing *M. tuberculosis* organisms

incomplete adjuvant containing *M. tuberculosis* organisms, µg/mouse	Antitoxin units per ml serum after injection of µg toxoid per mouse:-					
	1.25	2.5	5	10	20	40
2.5	9.6	4.8	4.8	4.8	38.4	38.4
	0.6	4.8	38.4	9.6	38.4	38.4
	0.6	4.8	4.8	0.6	19.2	38.4
	0.6	9.6	19.2	4.8	19.2	38.4
5	4.8	9.6	4.8	0.6	19.2	38.4
	4.8	4.8	4.8	19.2	4.8	38.4
	4.8	4.8	4.8	4.8	4.8	38.4
	9.6	4.8	4.8	4.8	38.4	38.4
10	0.6	1.2	0.6	4.8	38.4	76.8
	0.6	1.2	19.2	9.6	38.4	38.4
	0.6	0.6	4.8	19.2	4.8	38.4
	0.6	4.8	1.2	0.6	19.2	38.4
15	9.6	9.6	9.6	19.2	19.2	9.6
	2.4	19.2	9.6	19.2	9.6	9.6
	2.4	19.2	19.2	9.6	19.2	9.6
	1.2	4.8	9.6	9.6	19.2	19.2
30	2.4	9.6	9.6	19.2	19.2	19.2
	2.4	1.2	19.2	19.2	19.2	19.2
	1.2	0.6	19.2	38.4	76.8	19.2
	< 0.6	0.6	9.6	4.8	38.4	19.2
60	4.8	2.4	9.6	19.2	19.2	19.2
	2.4	4.8	19.2	19.2	19.2	19.2
	1.2	19.2	2.4	19.2	19.2	19.2
	1.2	2.4	4.8	19.2	19.2	19.2
500	4.8	2.4	4.8	9.6	9.6	9.6
	2.4	1.2	4.8	19.2	9.6	4.8
	2.4	1.2	1.2	4.8	4.8	2.4
	1.2	1.2	1.2	19.2	9.6	2.4

Table 6. Passive haemagglutination by Sera from Mice injected with Toxoid in Incomplete Adjuvant containing M. tuberculosis organisms

incomplete adjuvant containing M. tuberculosis organisms µg/mouse	Passive haemagglutination, reciprocal titre per 0.025 ml serum, after injection of µg toxoid per mouse:-					
	1.25	2.5	5	10	20	40
2.5	1	16	2	16	16	256
	2	4	4	4	256	128
	8	2	4	16	64	256
	1	2	32	16	4	256
5	4	64	8	16	64	128
	2	32	16	256	32	16
	4	8	32	64	64	64
	4	16	16	128	512	32
10	2	4	2	128	512	4
	1	10	1024	16	32	8
	-	1	4	64	64	16
	1	8	8	32	256	8
15	2	8	32	512	512	16
	-	4	32	64	256	32
	-	128	32	32	128	32
	2	2	128	128	256	128
30	2	1	-	256	64	-
	1	-	-	-	1	128
	-	1	1	-	2	128
	1	1	1	-	128	64
60	-	-	-	128	1024	8
	4	32	-	32	32	256
	4	1	1	128	256	128
	8	-	-	32	128	64
500	8	16	512	128	-	1
	8	2	16	128	128	512
	16	8	8	256	64	64
	8	2	16	64	32	64

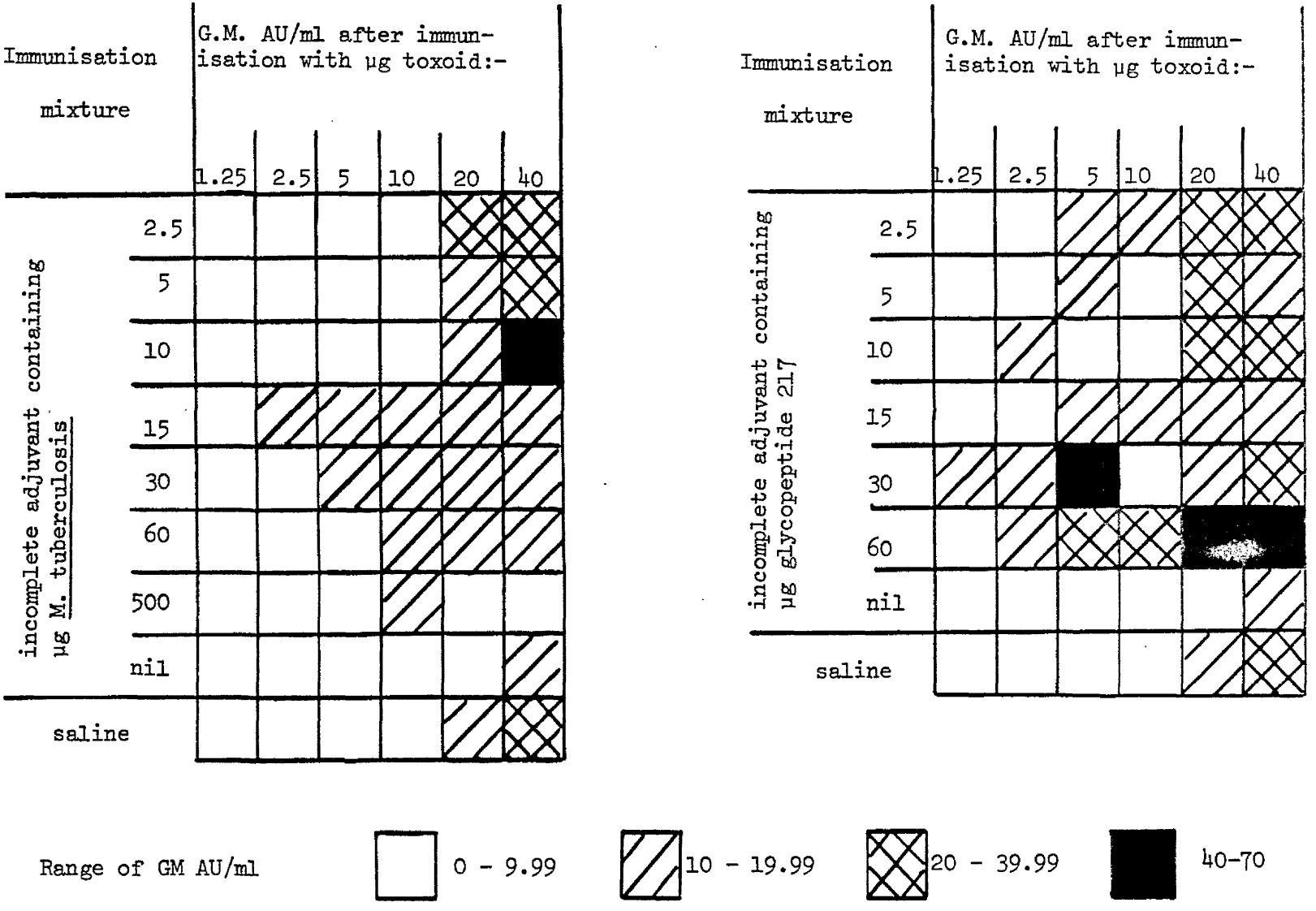
Table 7. Antitoxin Response to Injection of Toxoid in Incomplete Adjuvant containing Glycopeptide ST217

incomplete adjuvant containing glycopeptide ST217 µg/mouse	Antitoxin units per ml serum after injection of µg toxoid per mouse:- 1.25	2.5	5	10	20	40
	0.6	0.6	19.2	19.2	19.2	19.2
	1.2	4.8	19.2	19.2	38.4	19.2
2.5	0.6	4.8	19.2	19.2	38.4	38.4
	4.8	19.2	19.2	19.2	38.4	38.4
	9.6	< 0.6	19.2	9.6	19.2	38.4
	1.2	< 0.6	19.2	9.6	9.6	38.4
5	2.4	0.6	9.6	9.6	38.4	9.6
	1.2	< 0.6	9.6	9.6	38.4	9.6
	< 0.6	19.2	< 0.6	19.2	38.4	76.8
	< 0.6	19.2	< 0.6	19.2	38.4	38.4
10	< 0.6	9.6	< 0.6	9.6	19.2	19.2
	0.6	9.6	0.6	9.6	76.8	38.4
	9.6	9.6	19.2	9.6	19.2	19.2
	19.2	9.6	19.2	19.2	19.2	19.2
15	2.4	9.6	19.2	19.2	19.2	9.6
	2.4	9.6	9.6	19.2	19.2	9.6
	19.2	19.2	9.6	4.8	19.2	19.2
	4.8	19.2	4.8	9.6	19.2	19.2
30	19.2	9.6	4.8	9.6	19.2	38.4
	19.2	19.2	4.8	9.6	9.6	19.2
	9.6	9.6	38.4	38.4	38.4	38.4
	19.2	19.2	38.4	38.4	76.8	76.8
60	4.8	19.2	38.4	38.4	76.8	76.8
	9.6	19.2	19.2	38.4	38.4	76.8

Table 8. Passive haemagglutination by Sera from Mice injected with Toxoid in Incomplete Adjuvant containing Glycopeptide ST217

incomplete adjuvant containing glycopeptide ST217 µg/mouse	Passive haemagglutination reciprocal titre per 0.025 ml serum, after injection of µg toxoid per mouse:- 1.25	2.5	5	10	20	40
2.5	1	4	16	2	4	16
	1	8	32	2	4	1
	2	32	16	2	2	64
	1	2	16	2	1	16
5	4	-	4	4	8	64
	2	-	-	16	4	128
	1	1	1	4	64	128
	1	1	1	2	16	32
10	2	-	1	4	1	16
	4	-	1	4	16	16
	1	-	-	4	4	32
	2	-	-	1	64	64
15	1	32	32	512	128	1024
	8	128	64	32	128	128
	16	32	128	128	128	512
	32	32	128	128	256	256
30	2	16	8	32	256	128
	1	16	4	32	512	128
	16	16	8	64	128	512
	8	4	8	2048	128	64
60	16	128	256	2	2	16
	8	64	64	4	32	128
	16	32	128	16	8	128
	16	64	64	16	2	32

Figure 12. Graphical display of Antibody Response in Mice to Immunisation with graded doses of Toxoid in different Injection Mixtures



C. Inclusion of V. cholerae organisms in Toxoid Vaccines

The inclusion of 1 x 10^6 V. cholerae organisms per mouse in vaccines had little effect on the stimulation of the antitoxin response to toxoid in saline. In incomplete adjuvant there appeared to be a stimulated response where low toxoid concentrations, 1.25 μg and 2.5 μg, were injected together with 1 x 10^6 V. cholerae per mouse (Table 9).

The PHA titres obtained were generally low and showed no increased levels.

D. Complement-Fixing Antibacterial Antibody Response to Immunisation with V. cholerae organisms in Mice: Influence of Vaccine Content

The results indicated that the antibody response to injection of V. cholerae in saline was dependent on the number of organisms administered. The complement fixation titres of pooled sera from groups of ten animals are shown below:-

No. of V. cholerae organisms/mouse	10^3	10^4	10^5	10^6
Reciprocal complement fixation titre/0.025 ml	64	64	256	256

Since the complement fixation titre of serum from mice injected with 10^6 V. cholerae, against whole organisms, was 1/256 it was decided to explore the possible adjuvant effect of toxoid, known to contain small amounts of active toxin. Increased levels of complement fixing antibodies were not obtained by incorporating the organisms into any of the vaccine mixtures (Table 10). Indeed, in some instances the titre was considerably lower than 1/256, e.g. in the vaccine group injected with toxoid and V. cholerae organisms in saline.

Table 9. Comparison of Antitoxin Titres resulting from Immunisation with Toxoid, with and without the addition of V. cholerae organisms

Injection medium	µg Toxoid per mouse: 40		20		10		5		2.5		1.25	
	AU	PHA	AU	PHA	AU	PHA	AU	PHA	AU	PHA	AU	PHA
saline	76.8	256	19.2	512	9.6	32	9.6	8	2.4	2	4.8	4
	19.2	512	9.6	64	9.6	4	4.8	8	2.4	2	2.4	2
	19.2	512	9.6	16	9.6	16	4.8	4	1.2	2	1.2	8
	19.2	2048	9.6	16	9.6	32	4.8	1	1.2	2	1.2	-
saline + 10^6	19.2	32	19.2	16	19.2	16	9.6	64	1.2	4	1.2	16
	19.2	32	19.2	16	9.6	16	1.2	16	1.2	8	0.6	4
	19.2	256	19.2	16	9.6	16	4.8	128	1.2	8	1.2	2
V.cholerae/mouse	19.2	32	19.2	32	1.2	8	4.8	128	1.2	16	4.8	-
incomplete	19.2	512	9.6	256	9.6	128	9.6	2	4.8	2	< 0.6	1
	19.2	512	4.8	16	9.6	256	2.4	4	9.6	32	< 0.6	2
	19.2	512	4.8	32	9.6	64	1.2	2	4.8	64	< 0.6	-
adjuvant	19.2	16	4.8	4	9.6	32	0.6	2	1.2	2	< 0.6	1
incomplete	38.4	128	38.4	8	19.2	4	9.6	2	9.6	32	9.6	32
adjuvant + 10^6	38.4	8	9.6	8	1.2	2	9.6	8	9.6	16	9.6	8
V.cholerae/mouse	19.2	8	19.2	8	1.2	4	9.6	2	9.6	32	9.6	4
	38.4	2	9.6	8	1.2	2	9.6	8	9.6	8	9.6	8

AU = antitoxin units/ml serum

PHA = reciprocal titre/0.025 ml serum.

Table 10. Complement-Fixing Antibacterial Antibody Response to Immunisation with *V. cholerae* organisms in Mice : Influence of Vaccine Content

Injection Mixture per mouse	Reciprocal Complement Fixation Titre obtained on injection of µg Toxoid per mouse:-					
	40	20	10	5	2.5	1.25
1×10^6 *V. cholerae* in saline	256	32	64	32	128	32
	256	32	32	16	32	32
	64	32	32	16	64	128
	256	32	32	16	32	64
1×10^6 *V. cholerae* in incomplete adjuvant	64	128	64	128	64	256
	64	128	32	256	256	128
	64	128	128	128	64	128
	64	128	128	128	128	256
	or µg Mycobacterial glycopeptide, ST208, per mouse					
	40	20	10	5	2.5	1.25
1×10^6 *V. cholerae* in complete adjuvant	32	32	16	64	16	64
	128	64	32	16	512	256
	32	64	32	128	512	256
	128	16	32	128	256	256

The incorporation of V. cholerae organisms in water-in-oil emulsions containing mycobacterial glycopeptide adjuvant, ST208, did not stimulate the immune response, at least over the concentration range of ST208 studied.

7. Immunisation Studies in Guinea-pigs

Immunisation schedules in guinea-pigs were designed to study variables pertinent to cholera vaccination, (i) effect of using parenteral or oral immunisation routes, or a combination of the two; and (ii) effect of vaccine content. The results obtained are presented in the following sequence:-

I Serological tests, for antitoxin production, on sera from a preliminary group of guinea-pigs in which route of vaccine administration and vaccine content were studied.

II Serological tests, for antitoxin production, on sera and gut tissue extracts from guinea-pigs given 1, 2 or 3 doses of (a) toxoid or (b) toxoid + V. cholerae organisms. The primary dose given to these animals was administered either orally or i/m, and was incorporated in either incomplete adjuvant alone or containing mycobacterial glycopeptide. The 2ry and 3ry doses were oral and incorporated in incomplete adjuvant alone.

III Ileal loop tests, enterotoxin challenge, in guinea-pigs immunised with toxoid, by the schedules stated above.

IV Serological tests, for anti-bacterial antibody production, on sera from guinea-pigs immunised with (a) toxoid + V. cholerae organisms or (b) V. cholerae organisms, by the schedules mentioned above.

V Ileal loop tests, viable V. cholerae challenge, in guinea-pigs immunised with (a) toxoid, (b) toxoid + V. cholerae organisms or (c) V. cholerae organisms, by the above schedules.

A. Immunisation with Cholera Toxoid in a Liquid Paraffin-Gelatin Base

In the first experiment, groups of four guinea-pigs were given vaccine containing cholera toxoid in 15% gelatin mixed with innocuous liquid paraffin or saline, orally. There was no stimulation of serum antitoxin levels with or without the addition of 250 μg of mycobacterial glycopeptide ST217, or 250 μg of M. tuberculosis DT whole organisms (Table 11).

B. Immunisation with a Single Intramuscular Injection of Toxoid in Incomplete Adjuvant Supplemented with Glycopeptides or Whole Mycobacteria

The incorporation of 250 μg mycobacterial glycopeptides, ST217 or ST208, or M. tuberculosis strain DT organisms into the water-in-oil emulsion containing toxoid produced a significant stimulation of cholera antitoxin response after a single i/m injection (Table 12). Poor responses were obtained with toxoid vaccines in incomplete adjuvant or in a complete adjuvant incorporating glycopeptide ST210.

C. Oral and/or Intramuscular Primary Immunisation with Toxoid, plus One Oral Booster Dose (G.P. 303-320)

In this group of guinea-pigs it was found that two oral doses did not produce increased levels of serum antitoxin (Table 13). There was no difference in the responses obtained with toxoid given orally in incomplete adjuvant with or without the addition of mycobacterial glycopeptide, ST208. It was noted, however, that detectable levels of antitoxic activity were obtained with ammonium sulphate precipitates of extracts of small intestines of these animals. The levels of antiblueing activity in the ammonium sulphate precipitates were similar in all groups

Table 11. Oral Administration of Cholera Toxoid in 15% Gelatin mixed with Liquid Paraffin or Saline

Guinea-pig code no.DSTGP	Vaccine	antitoxin units/ml serum	PHA reciprocal titre/0.025 ml serum
256-259	50 µg toxoid/liquid paraffin/ gelatin	< 0.6	2
		< 0.6	2
		< 0.6	4
		< 0.6	-
260-263	50 µg toxoid/saline/gelatin	< 0.6	-
		< 0.6	4
		< 0.6	1
		< 0.6	-
264-267	50 µg toxoid/liquid paraffin/ 250 µg ST217/gelatin	< 0.6	-
		< 0.6	-
		< 0.6	-
268-271	50 µg toxoid/saline/ 250 µg ST217/ gelatin	< 0.6	-
		< 0.6	-
		< 0.6	-
		< 0.6	1
272-275	50 µg toxoid/liquid paraffin/ 250 µg *M. tuberculosis* organisms/ gelatin	< 0.6	-
		< 0.6	-
		< 0.6	-
		< 0.6	-
276-279	50 µg toxoid/saline/250 µg *M. tuberculosis* organisms/gelatin	< 0.6	-
		< 0.6	4
		< 0.6	-
		< 0.6	-

Table 12. Intramuscular injection of Cholera Toxoid in Incomplete and Complete Adjuvant Mixtures

Guinea-pig code no.DSTGP	Vaccine	antitoxin units per ml	reciprocal of PHA titre
280-283	60 µg Toxoid in 0.1 ml IA, i/m	4.8	8
		< 0.6	1
		0.6	2
		0.6	-
284-287	60 µg Toxoid + 250 µg ST217 in 0.1 ml IA, i/m	4.8	8
		153.6	4096
		1.2	512
		0.6	1024
288-291	60 µg Toxoid + 250 µg ST208 in 0.1 ml IA, i/m	38.4	128
		38.4	2048
		38.4	512
		1.2	32
292-295	60 µg Toxoid + 250 µg ST210 in 0.1 ml IA, i/m	0.6	8
		0.6	4
		0.6	-
		0.6	32
296-301	60 µg Toxoid + 250 µg *M. tuberculosis* D.T. in 0.1 ml, i/m	38.4	512
		9.6	32
		9.6	64
		153.6	64
		9.6	4
		0.6	8

IA = incomplete adjuvant: Esso Bayol F, 0.5 ml;

Arlacel A, 0.2 ml; toxoid, 0.3 ml.

Table 13. Intramuscular and/or oral primary administration of toxoid in incomplete or complete adjuvant, followed by an oral booster dose

Guinea-pig code no.DSTGP	Primary Immunisation	serum antitoxin units/ml	reciprocal of serum PHA titre/ 0.025 ml	gut extract antitoxin level (AU/ml)
303-305	60 µg toxoid in IA, in oral capsule	< 0.6	4	1.2
		< 0.6	4	1.2
		< 0.6	-	1.2
306-308	100 µg toxoid in IA, i/m	4.8	32	0.6
		19.2	32	2.4
		1.2	8	0.6
309-311	60 µg toxoid + 300 µg ST208 in IA, in oral capsule	< 0.6	1	1.2
		< 0.6	1	1.2
		< 0.6	1	1.2
312-314	100 µg toxoid + 500 µg ST208, i/m	4.8	2	0.6
		1.2	8	1.2
		0.6	4	1.2
315-317	60 µg toxoid in IA, in oral capsule and 100 µg toxoid in IA, i/m	9.6	64	1.2
		0.6	1	1.2
		4.8	16	0.6
318-320	60 µg toxoid + 300 µg ST208 in IA in oral capsule and 100 µg toxoid + 500 µg ST208 in IA, i/m	4.8	8	1.2
		4.8	64	0.6
		1.2	16	1.2

IA = incomplete adjuvant: Octadecane, 2.5 ml; Bayol F, 2.5 ml; Arlacel, 1.0 ml; saline, 2.5 ml; Wellcome toxoid, 1.0 ml.

All animals received an oral booster of a capsule containing 60 µg toxoid in 0.3 ml IA 2 months after the primary inoculation.

of animals and were not affected by the presence or absence of glycopeptide ST208 in the vaccines.

In addition, these ammonium sulphate precipitates produced precipitin lines against sheep anti-guinea-pig globulins in gel diffusion tests (Plate 10).

Serum antitoxin was produced in response to i/m injection of toxoid in incomplete adjuvant, however, no stimulatory effect of the glycopeptide ST208 was noted. There also appeared to be no advantage in administering an oral dose of vaccine simultaneously with the i/m primary injection.

D. Primary Oral or Intramuscular Immunisation with Toxoid: Effect of Oral Booster Doses

The results of both the neutralisation of intradermal blueing and the PHA test demonstrated that no serum antitoxin response was obtained after 1, 2 or 3 oral administrations of toxoid in incomplete adjuvant, with or without the addition of ST208. However, serum cholera antitoxin levels were detectable throughout the 3 month period of the test in guinea-pigs immunised by an i/m primary inoculation of toxoid. The incorporation of mycobacterial glycopeptide ST208 into the i/m primary dose resulted in a slight increase in the levels of serum antitoxin (Table 14).

E. Measurement of Gut-Associated Antitoxin in Guinea-pigs Immunised with Toxoid

A gut-associated antibody response after i/m immunisation with cholera toxoid in incomplete adjuvant alone or incorporating glycopeptide ST208, was poor even after one or two oral booster doses. Some antitoxin

Plate 10. Gel diffusion precipitin tests with gut tissue extracts and sheep anti-guinea-pig immunoglobulin.

The sera were tested in duplicate against rabbit anti-guinea-pig serum in the central well of the left unit and sheep anti-guinea-pig serum in the central of the right unit.

1 - 6 Normal guinea-pig

7 - 36 Inoculated guinea pigs Nos. 303, 320, 333, 334, 335, 356, 357, 358, 377, 378, 379, 399, 400 respectively.

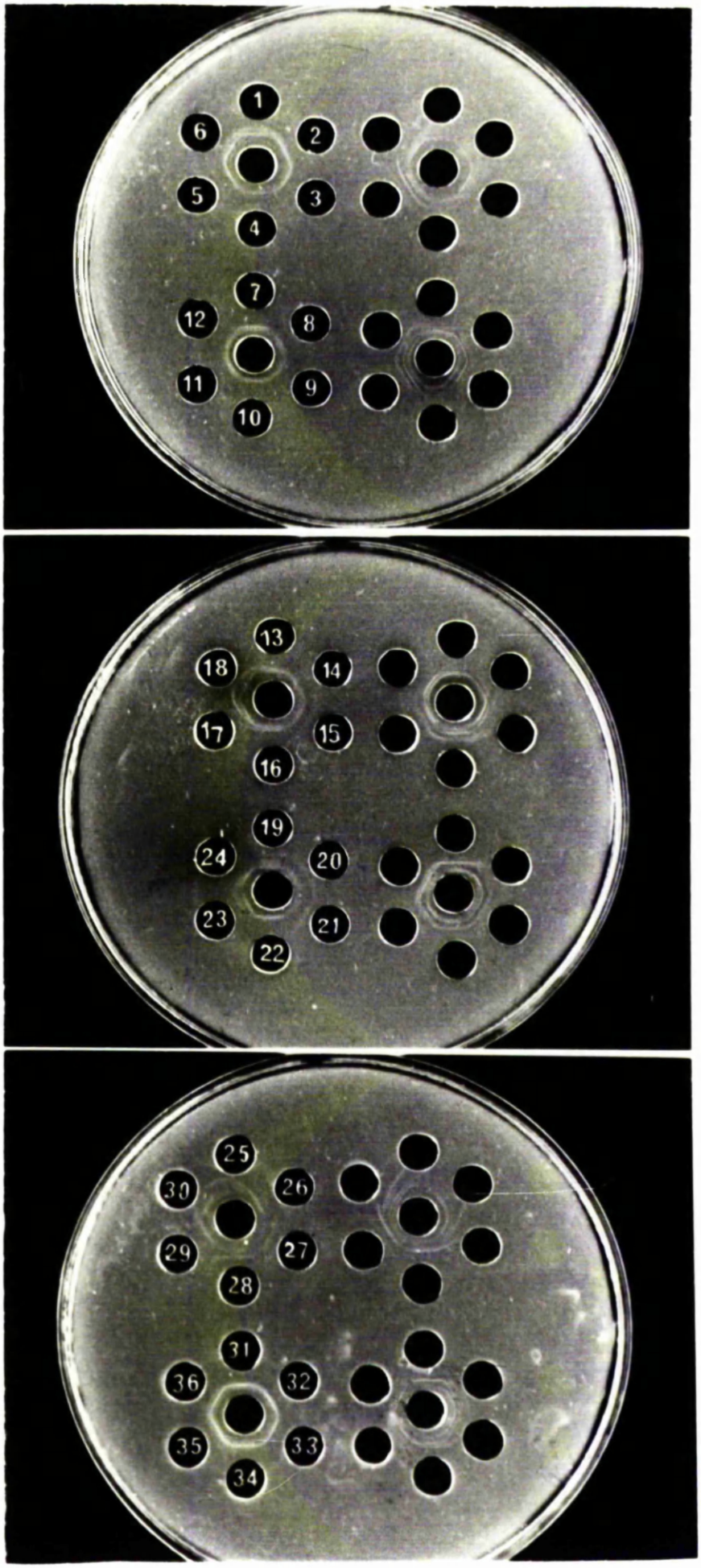

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36

Table 14. Serum antitoxin response to oral or intra-muscular immunisation with toxoid in incomplete or complete adjuvant, and the effect of oral booster doses of toxoid in incomplete adjuvant

Guinea-pig code no. DST GP	Primary immunisation	Serum antitoxin level after:- 1 month, no booster		2 months, 1x0.3ml booster after 6 weeks		3 months, 2x0.3ml boosters after 6 & 10 weeks	
		AU	PHA	AU	PHA	AU	PHA
333-343	60 µg toxoid in IA, oral capsule	< 0.6	-	< 0.6	-	< 0.6	-
		< 0.6	-	< 0.6	-	< 0.6	1
		< 0.6	-	< 0.6	-	< 0.6	1
		< 0.6	-	< 0.6	-	< 0.6	-
356-364	60 µg toxoid + 300 µg ST208 in IA, oral capsule	< 0.6	-	< 0.6	1	< 0.6	1
		< 0.6	1	< 0.6	1	< 0.6	2
		< 0.6	1	< 0.6	-		
377-385	100 µg toxoid in IA, i/m	76.8	256	38.4	64	38.4	16
		38.4	32	38.4	128	38.4	256
		38.4	8	38.4	2	38.4	256
398-406	100 µg toxoid + 500 µg ST208 in IA, i/m	76.8	256	76.8	32	76.8	8
		38.4	256	76.8	64	76.8	1024
		153.6	32	153.6	64	76.8	256

AU = antitoxin units/ml of serum, determined by the intradermal anti-blueing test

PHA = reciprocal of passive haemagglutination titre/0.025 ml of serum

IA = incomplete adjuvant: Octadecane, 2.5 ml; Bayol F, 2.5 ml; Arlacel, 1.0 ml; saline, 2.5 ml; toxoid 1.0 ml.

was detected, in the intradermal blueing inhibition test, where a single oral dose of toxoid was administered, but the presence of ST208 did not seem to increase the effect (Table 15).

F. Primary Oral or Intramuscular Immunisation with Toxoid and *V. cholerae* organisms: Effect of Oral Booster Doses

Serum antitoxin was not detected where a purely oral immunisation schedule, using water-in-oil emulsions containing cholera toxoid and *V. cholerae* organisms, was used. However, where i/m primary immunisation was used, levels of serum antitoxin were detected throughout the three month period of observation (Table 16). The results did not indicate that the incorporation of ST208 in the vaccine stimulated an increased antitoxin response.

G. Measurement of Gut-associated Antitoxin in Guinea-pigs Immunised with Toxoid plus *V. cholerae* organisms

Low levels of cholera enterotoxin-neutralising antibodies were detected in this group of guinea-pigs (Table 17). This response was similar to that obtained with guinea-pigs immunised with toxoid alone (Table 15).

H. Ouchterlony Gel Diffusion Test

(i) Determination of Optimum Antigen Concentration

A range of N.I.H. toxin concentrations in physiological saline (100 µg, 200 µg, 300 µg, 400 µg, 500 µg, 1.0 mg, 2.0 mg, 3.0 mg, 4.0 mg, 6.0 mg and 10 mg/ml) were tested against the following guinea-pig antisera:-

Table 15. Gut-associated antitoxin response to oral or intra-muscular immunisation with toxoid in incomplete or complete adjuvant, and the effect of oral booster doses of toxoid in incomplete adjuvant

Guinea-pig code no. DST GP	Primary immunisation	Gut extract antitoxin level after:- 1 month	2 months	3 months
		no booster	1 x 0.3 ml booster after 6 weeks	2 x 0.3 ml boosters after 6 & 10 weeks
333-343	60 µg toxoid in IA, oral capsule	0.6	2.4	0.3
		0.6	< 0.3	< 0.3
		2.4	< 0.3	< 0.3
		2.4	< 0.3	< 0.3
377-385	100 µg toxoid in IA, i/m	< 0.3	< 0.3	0.3
		< 0.3	0.3	0.3
		< 0.3	0.3	< 0.3
356-364	60 µg toxoid + 300 µg ST208 in IA, oral capsule	2.4	< 0.3	0.3
		2.4	< 0.3	< 0.3
		0.6	< 0.3	NT
398-406	100 µg toxoid + 500 µg ST208, i/m	< 0.3	< 0.3	< 0.3
		< 0.3	< 0.3	< 0.3
		0.3	0.3	0.3

IA = incomplete adjuvant: Octadecane, 2.5 ml; Bayol F, 2.5 ml; Arlacel, 1.0 ml; saline, 2.5 ml; toxoid, 1.0 ml.

Table 16. Serum antitoxin response to oral or intra-muscular immunisation with toxoid and *V. cholerae* organisms in incomplete or complete adjuvant, and the effect of oral booster doses of toxoid and *V. cholerae* organisms in incomplete adjuvant

Guinea-pig code no. DST GP	Primary immunisation	Serum antitoxin levels after:- 1 month, no booster		2 months, 1 x 0.3 ml booster after 6 weeks		3 months, 2 x 0.3 ml boosters after 6 & 10 weeks	
		AU	PHA	AU	PHA	AU	PHA
470-478	60 µg toxoid with	< 0.6	2	< 0.6	1	< 0.6	2
	6×10^4 *V.cholerae*	< 0.6	-	< 0.6	-	< 0.6	-
	in IA, oral capsule	N.T.	N.T.	< 0.6	16	< 0.6	-
512-520	60 µg toxoid with	< 0.6	8	< 0.6	1	< 0.6	2
	6×10^4 *V.cholerae*	< 0.6	8	< 0.6	2	< 0.6	-
	and 300 µg ST208, in IA, oral capsule	< 0.6	4	N.T.	N.T.	< 0.6	-
491-499	100 µg toxoid with	4.8	128	9.6	1024	9.6	256
	10^5 *V.cholerae* in IA,	4.8	64	9.6	32768	9.6	16
	i/m	2.4	8	N.T.	N.T.	9.6	4096
533-541	100 µg toxoid with	4.8	16	76.8	256	2.4	-
	10^5 *V.cholerae* and	9.6	32	9.6	64	< 0.6	1
	500 µg ST208 in IA,i/m	9.6	32	N.T.	N.T.	9.6	16

AU = antitoxin units/ml of serum, determined by the intradermal anti-blueing test

PHA = reciprocal of passive haemagglutination titre/0.025 ml of serum

IA = incomplete adjuvant: Octadecane, 2.5 ml; Bayol F, 2.5 ml; Arlacel, 1.0 ml; 1.9×10^6 *V.cholerae* organisms in saline, 2.5 ml; toxoid, 1.0 ml

N.T. = animal died, during period of experiment.

Table 17. Gut-associated antitoxin response to oral or intra-muscular immunisation with toxoid and *V. cholerae* organisms in incomplete or complete adjuvant, and the effect of oral booster doses of toxoid and *V. cholerae* organisms in incomplete adjuvant

Guinea-pig code no. DST GP	Primary immunisation	Gut extract antitoxin level after:- 1 month	2 months	3 months
		no booster	1 x 0.3 ml booster after 6 weeks	2 x 0.3 ml boosters after 6 & 10 weeks
470-478	60 µg toxoid with 6×10^4 *V. cholerae* in IA, oral capsule	0.3	< 0.3	< 0.3
		0.3	< 0.3	< 0.3
		N.T.	< 0.3	0.3
491-499	100 µg toxoid with 10^5 *V. cholerae* in IA, i/m	< 0.3	0.6	0.3
		< 0.3	0.6	< 0.3
		< 0.3	N.T.	0.3
512-520	60 µg toxoid with 6×10^4 *V. cholerae* and 300 µg ST208, oral capsule	0.3	0.3	0.3
		0.3	0.3	< 0.3
		< 0.3	N.T.	< 0.3
533-541	100 µg toxoid with 10^5 *V. cholerae* and 50 µg ST208, i/m	< 0.3	0.6	< 0.3
		< 0.3	< 0.3	< 0.3
		< 0.3	N.T.	0.3

IA = incomplete adjuvant: Octadecane, 2.5 ml; Bayol F, 2.5 ml; Arlacel, 1.0 ml; 1.9×10^6 *V. cholerae* organisms in saline, 2.5 ml; toxoid, 1.0 ml

N.T. = animal died during period of experiment.

Guinea-pig Code No. DST GP	Vaccine	Route of administration
281 283	60 µg toxoid in incomplete adjuvant	i/m
289	60 µg toxoid + 250 µg ST208 in incomplete adjuvant	i/m
298 300	60 µg toxoid + 250 µg *M. tuberculosis* DT in incomplete adjuvant	i/m

No precipitin lines were seen with any of the antisera against any of the toxin dilutions employed.

Serial dilutions of the toxoid preparation (neat - 1/32) were tested against the antiserum numbers used above. Lines of precipitation were seen against serum numbers 289, 298 and 300 against the neat toxoid. The lines became decreasingly distinct with the 1/2 and 1/4 dilutions and were absent with the higher dilutions.

The 1/2 dilution of toxoid was chosen for the study of all guinea-pig sera raised against cholera toxoid. The results of precipitation are shown on Plate 11 a and b.

(ii) Precipitation between Toxoid and Guinea-pig Antisera, in Ouchterlony Double Gel Diffusion

It was apparent that only guinea-pigs immunised i/m with preparations containing toxoid stimulated a sufficient level of antitoxin to give a positive Ouchterlony test against toxoid (Table 18). In no case did precipitation occur with serum from guinea-pigs immunised by a purely oral regime.

Plate 11 a & b. Gel diffusion precipitin tests with guinea-pig sera and toxoid.

Tox - Wellcome cholera toxoid

Numbers - Guinea-pig experimental numbers

(a)

(b)

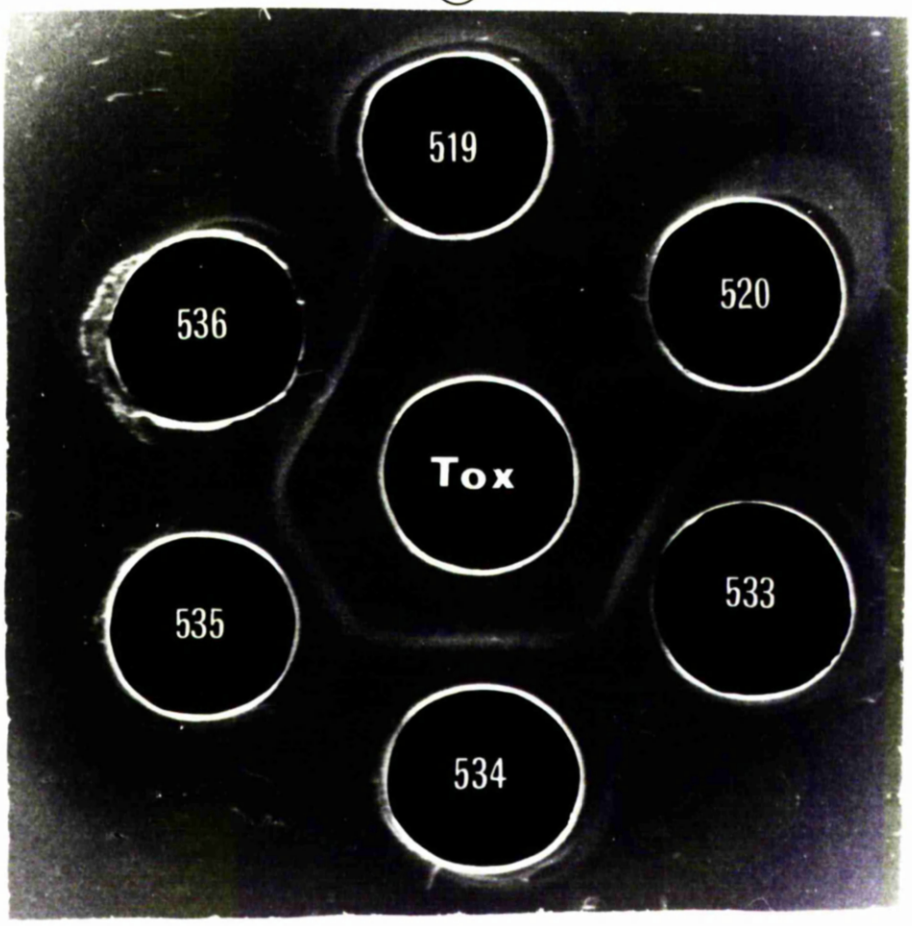


Table 18. Ouchterlony double diffusion in gel; testing of guinea-pig antisera against toxoid

Guinea-pig Code No. DST GP	Immunisation (i/m): 60 µg toxoid in IA + 250 µg adjuvant:-	no. of +ve sera / no. of -ve sera for precipitin line formation	G.M. A.U./ml (& range)
280-283	nil	4/4	< 3.3(<0.6-4.8)
284-287	ST217	4/4	4.8(0.6-153.6)
288-291	ST208	4/4	16.2(1.2-38.4)
292-295	ST210	1/4	0.6(nil)
296-301	M. tuberculosis DT	6/6	12.0(0.6-153.6)
	Primary immunisation (i/m): 100 µg toxoid in IA with octadecane + additions:-		
318-320	500 µg ST208. Oral dose of 60 µg toxoid in IA with octadecane and 300 µg ST208. Oral booster x 1.	2/3	3.0(1.2-4.8)
377-379	nil	3/3	49.0(38.4-76.8)
380-382	Oral booster x 1.	3/3	38.4(nil)
383-385	Oral boosters x 2.	3/3	38.4(nil)
398-400	500 µg ST208	3/3	77.6(38.4-153.6)
401-403	500 µg ST208 Oral booster x 1.	3/3	97.7(76.8-153.6)
404-406	500 µg ST208 Oral boosters x 2.	3/3	76.8(nil)
	Primary immunisation (i/m): 100 µg toxoid and 10^5 V. cholerae in IA with octadecane + additions:-		
491-493	nil	1/3	3.8(2.4-4.8)
494-495	Oral booster x 1.	2/2	9.6(nil)
533-535	500 µg ST208.	3/3	7.6(4.8-9.6)
536-537	500 µg ST208 Oral booster x 1.	1/2	27.5(9.6-76.8)

In the first group of animals, GP280-301, which were immunised with 60 μg toxoid in incomplete adjuvant supplemented with either mycobacterial cells or a glycopeptide preparation, it was noteworthy that only one antiserum from the group receiving glycopeptide ST210 gave a precipitin line which was in agreement with the results of the i/d blueing inhibition test (Table 12). In general, the gel diffusion precipitin test results paralleled the antitoxin units/ml.

I. Fluid Accumulation in Guinea-pig Ileal loops in Response to Challenge with Cholera Enterotoxin, after Immunisation with Toxoid Vaccines

(i) Choice of Measurement

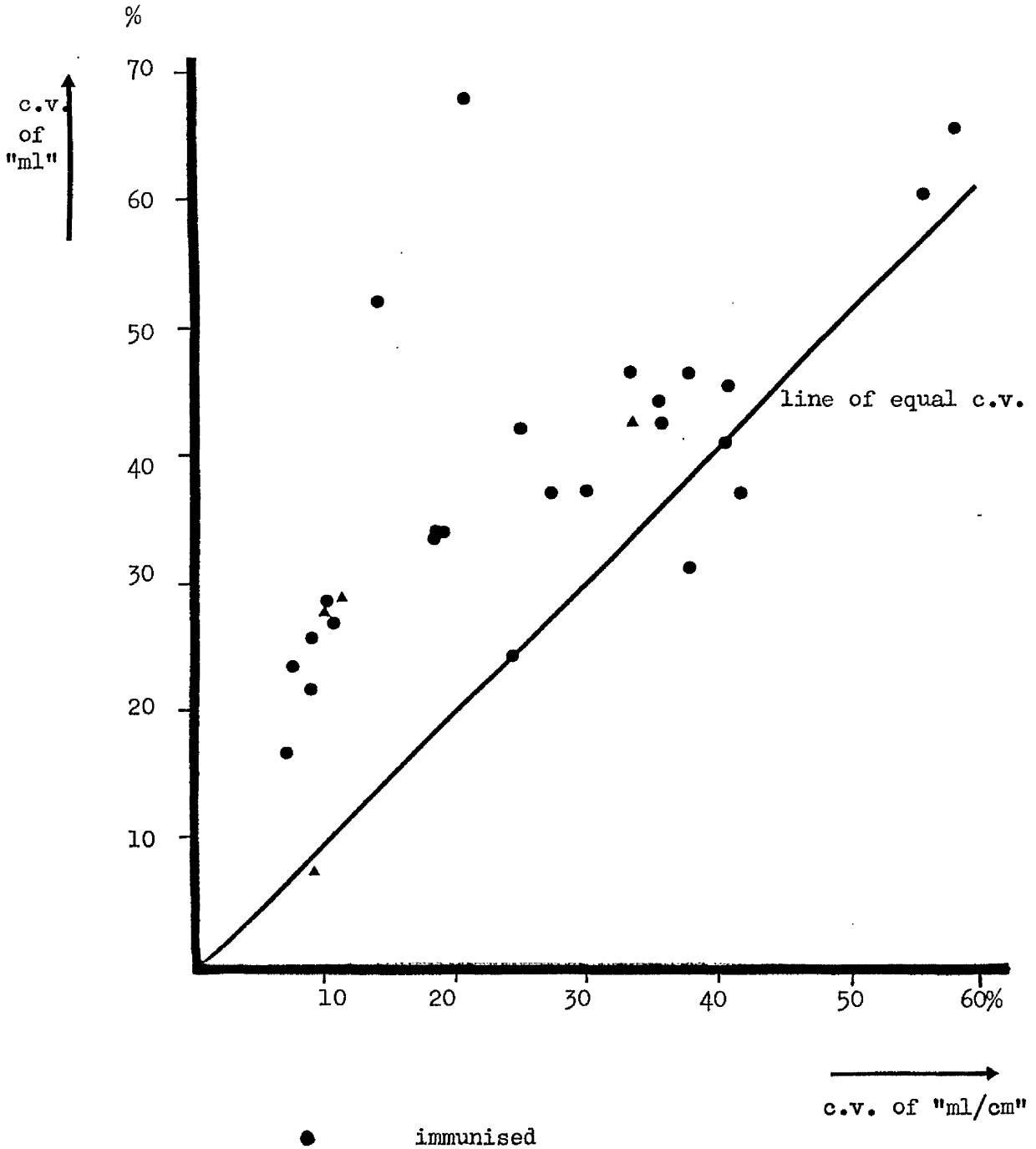
In all ileal loop tests fluid accumulation was recorded both as a value of ml/loop and ml/cm of loop. As discussed later (p. 168) the relative advantages of the two measurements were questioned. The coefficients of variation (c.v.) of the "ml" values and the "ml/cm" values obtained in guinea-pig groups shown in Appendix 2, Table 2, were calculated, i.e.

$$\text{c.v.} = \frac{100s}{\bar{x}} \%$$

for each group of 4 ileal loops challenged with the same amount of toxin, within each immunisation group.

These results were plotted on Fig 13. If the same degree of variability was associated with each of these measurements, the points on the graph would fall along a line at 45° to the axes. However, the points were found to lie distinctly to the left of this line, indicating that a greater degree of variability was associated with the measurement of "ml" fluid accumulated per ileal loop.

Figure 13. Comparison of the Coefficients of Variation (c.v.) of the two methods of assessing Fluid Accumulation in Ileal Loops



The "ml/cm" results were therefore chosen as the best indication of fluid accumulation and are presented throughout this thesis.

(ii) Fluid Accumulation in Ileal Loops

The results showed that the accumulation of isotonic fluid in guinea-pig ileal loops could be reduced by immunisation. No reduction was apparent in guinea-pigs given a single oral immunisation of either toxoid in incomplete adjuvant alone (DST GP 321-324, Appendix 2, Table 1) or in incomplete adjuvant + ST208 (DST GP 344-347, Appendix 2, Table 1). Where either of these oral primary doses was followed by two oral administrations of toxoid in incomplete adjuvant alone no protection against ileal swelling was seen (DST GP 329-332 and 352-355, Appendix 2, Table 1). However where the primary dose was followed by only one oral booster, protection was seen in both cases (DST GP 325-328 and 348-351, Appendix 2, Table 1), although this was more marked where the primary inoculum was toxoid in incomplete adjuvant containing ST208.

In guinea-pigs which had received i/m primary inoculations of toxoid in incomplete adjuvant alone (Appendix 2, Table 2), a decrease in fluid accumulation was noted only where the primary injection was followed with one oral booster of toxoid in incomplete adjuvant (DST GP 369-372). Where no or two oral boosters were given no decrease was noted (DST GP 365-368 and 373-376). However, where the primary injection mixture was toxoid in incomplete adjuvant containing ST208, reductions in fluid accumulation were evident in all three vaccine groups, primary injection alone or plus one or two oral boosters (DST GP 386-397).

The results shown in Appendix 2, Tables 1 and 2, were analysed using a student t-test (Appendix 4). The t-test results are shown in

Tables 19 and 20. Only the group of guinea-pigs which received an i/m dose of toxoid in incomplete adjuvant containing glycopeptide ST208, is convincingly protected from ileal challenge with enterotoxin, when both analyses A and B are considered.

Figures 14 and 15 show a graphical comparison of the mean fluid accumulation in groups of immunised and unimmunised guinea-pigs. Groups of animals in which the fluid accumulation is highly significantly reduced compared to the unimmunised value (Analysis B, Appendix 4) are indicated. There is a close similarity between the groups mentioned above, in which the raw data indicate a decrease in fluid accumulation, and the groups which show highly significant decreases, compared to control values, on Figs 14 and 15.

J. Agglutination of whole V. cholerae cells by Sera from Animals Immunised with Vaccines containing V. cholerae organisms

Sera from all guinea-pigs immunised with whole V. cholerae were tested using the microtitration assay. It was not possible to test gut-tissue extracts due to their floccular nature, since buttons of unagglutinated vibrios could not be distinguished.

It was possible to obtain only very low titres, usually 1/2 or 1/4.

K. Agglutination of Vibrio "H" and "O" Cell Suspensions by Selected Antisera

Guinea-pig antisera were selected from animals which had received whole V. cholerae organisms in an immunisation schedule known to be efficient in serum antitoxin production. The agglutination titres were recorded (Table 21).

Table 19. Statistical Analysis of ileal loop test results - Toxin Challenge

Analysis A. Based on difference in "ml/cm" values between challenge and control loops.

Analysis B. Based on difference in "ml/cm" values between loops in immunised and unimmunised animals.

+ indicates significant protection from challenge.
- indicates no significant protection.

Guinea-pig code no. DST GP	Vaccine	Route of administration	Time months	Response to cholera toxin (mg): 0.25 A	0.25 B	0.5 A	0.5 B	1.0 A	1.0 B	0 A	0 B
321 322 323 324	60 µg Toxoid in IA	oral x 1 0.3 ml	1	+	-	±	±	±	±	.	-
325 326 327 328	60 µg Toxoid in IA + booster after 6 weeks	oral x 2 0.3 ml	2	±	+	±	+	±	+	.	+
329 330 331 332	60 µg Toxoid in IA + boosters after 6 & 10 weeks	oral x 3 0.3 ml	3	-	-	-	-	-	±	.	+
344 345 346 347	60 µg Toxoid with 300 µg ST208 in IA	oral x 1 0.3 ml	1	+	-	+	-	+	-	.	-
348 349 350 351	60 µg Toxoid with 300 µg ST208 in IA + booster after 6 weeks 60 µg Toxoid in IA	oral x 2 0.3 ml	2	-	+	-	+	-	+	.	+
352 353 354 355	60 µg Toxoid with 300 µg ST208 in IA + boosters after 6 & 10 weeks, 60 µg Toxoid in IA	oral x 3 0.3 ml	3	-	+	-	+	-	+	.	+

See Appendix 2, Table 1 for raw data. See Appendix for details of statistical analyses.

Table 20. Statistical Analysis of ileal loop test results - Toxin Challenge

Analysis A. Significance of difference in "ml/cm" values between challenge and control loops.

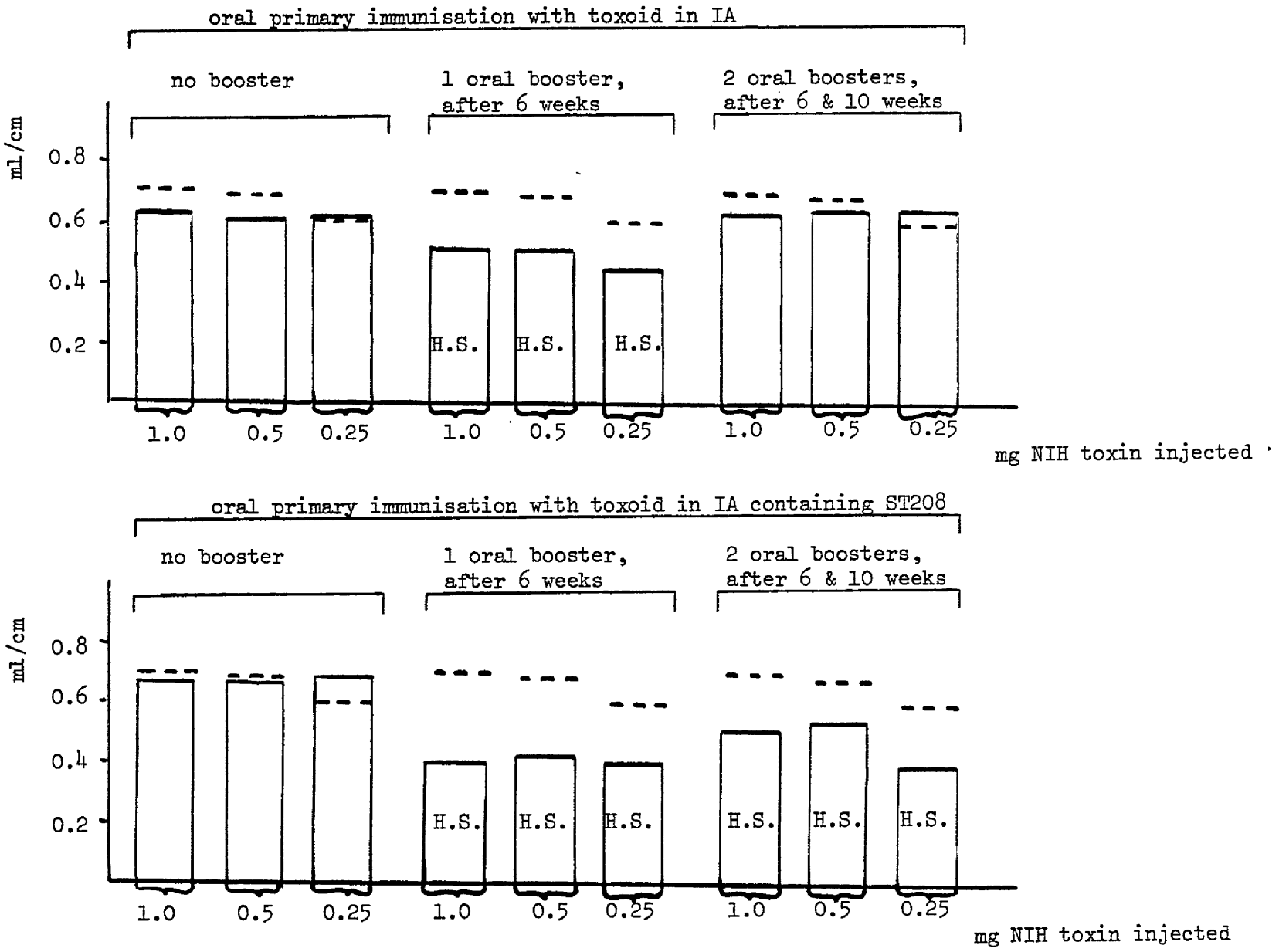
Analysis B. Significance of difference in "ml/cm" values between loops in immunised and unimmunised animals.

+ indicates significant protection from challenge
- indicates no significant protection.

Guinea-pig Code No. DST GP	Vaccine	Route of administration	Time months	Response to cholera toxin (mg): 0.25 A	0.25 B	0.5 A	0.5 B	1.0 A	1.0 B	0 A	0 B
365 366 367 368	100 µg Toxoid in IA	i/m x 1 0.5 ml	1	+	-	+	-	+	+	.	-
369 370 371 372	100 µg Toxoid in IA + booster after 6 weeks, 60 µg Toxoid in IA	i/m x 1 0.5 ml; oral x 2 0.3 ml	2	+ -	+	+ -	+	+ -	+	.	+
373 374 375 376	100 µg Toxoid in IA + boosters after 6 & 10 weeks, 60 µg Toxoid in IA	i/m x 1 0.5 ml; oral x 2 0.3 ml	3	-	-	-	-	-	-	.	-
386 387 388 389	100 µg Toxoid with 500 µg ST208 in IA	i/m x 1 0.5 ml	1	+	+	+	+	+	+	.	+
390 391 392 393	100 µg Toxoid with 500 µg ST208 in IA + booster after 6 weeks 60 µg Toxoid in IA	i/m x 1 0.5 ml; oral x 1 0.3 ml	2	+	+	+	+	+	+	.	+
394 395 396 397	100 µg Toxoid with 500 µg ST208 in IA + boosters after 6 & 10 weeks; 60 µg Toxoid	i/m x 1 0.5 ml; oral x 2 0.3 ml	3	+	+	+	+	+	+	.	+
Controls 628, 638-642	Non-immunized			+	.	-	.	-	.	.	-

See Appendix 2, Table 2 for raw data. See Appendix 4 for details of statistical analyses.

Figure 14. Graphical representation of decrease in fluid accumulation in ileal loops after immunisation with toxoid

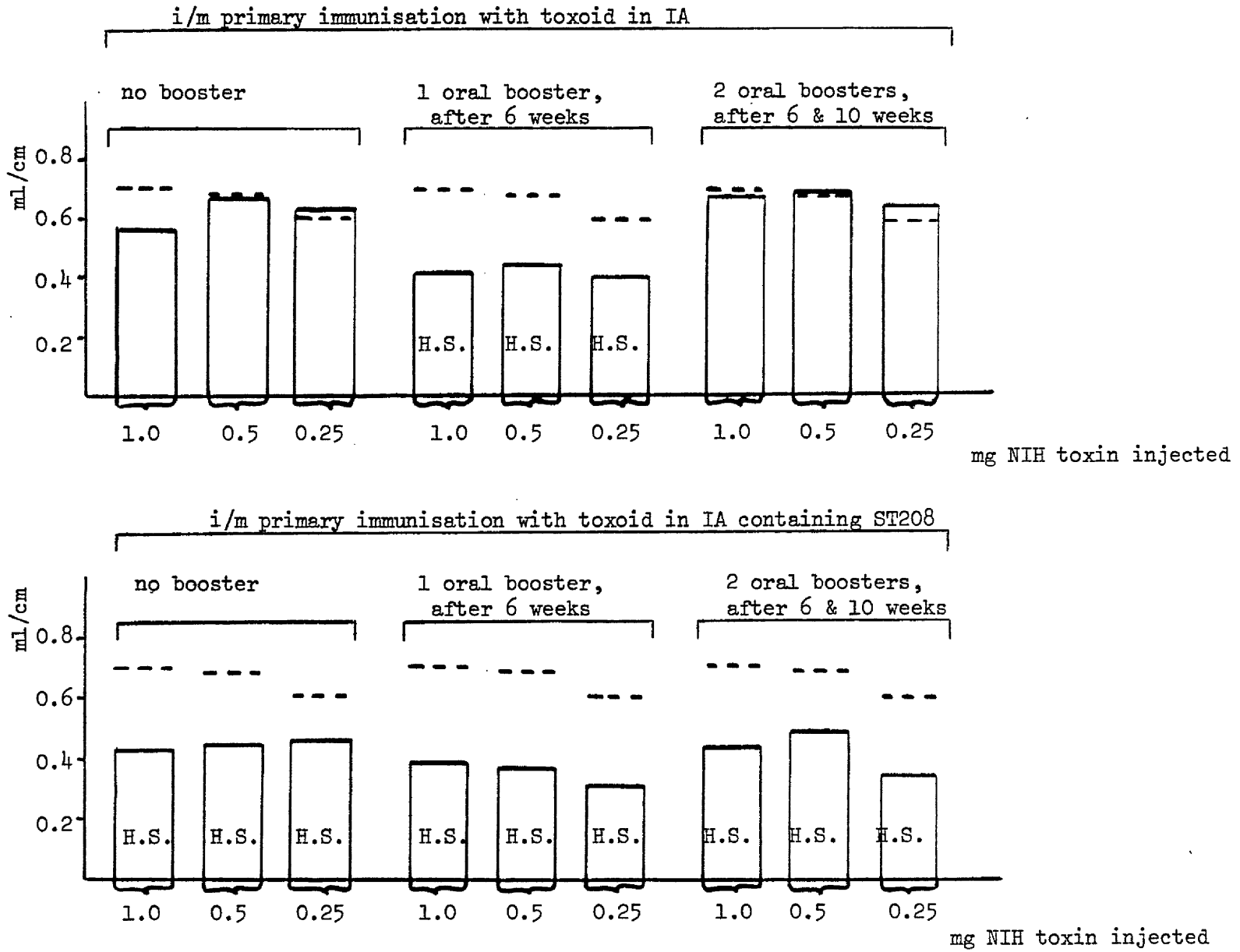


——— $\bar{x}$ of 4 immunised guinea-pigs

– – – $\bar{x}$ of 6 unimmunised guinea-pigs

H.S. = highly significant reduction compared with unimmunised guinea-pigs

Figure 15. Graphical representation of decrease in fluid accumulation in ileal loops after immunisation with toxoid



$\bar{x}$ of 4 immunised guinea-pigs

$\bar{x}$ of 6 unimmunised guinea-pigs

HS = highly significant reduction compared with unimmunised guinea-pigs

Table 21. Agglutination of vibrio "H" and "O" cell suspensions

Immunisation regime	Guinea-pig Code No. DST GP	Serum dilution: neat H	neat O	1/2 H	1/2 O	1/4 H	1/4 O	1/8 H	1/8 O	1/16-1/152 H	1/16-1/152 O
1 x 10^5 V.cholerae in incomplete adjuvant, i/m	575	+++	-	++	-	+	-	+	-	-	-
	576	+++	-	+++	-	+	-	+	-	-	-
1 x 10^5 V.cholerae in incomplete adjuvant + 500 µg ST208, i/m	617	+++	-	+++	-	++	-	+	-	-	-
	618	++	-	++	-	+	-	+	-	-	-

No agglutination of the "O" suspension occurred with the test sera, however agglutination of the "H" suspension was noted at a 1/8 serum dilution.

L. Complement-fixing Antibody Response to Immunisation with *V. cholerae* organisms Alone or plus Toxoid

Complement-fixing anti-*V. cholerae* antibodies were detected in low titres in the sera of all animals given *V. cholerae*-containing vaccines (Table 22). No relative advantage of oral or i/m primary inoculation, or for the inclusion of glycopeptide ST208 was observed. The response obtained where *V. cholerae* alone was used for immunisation appeared to be slightly higher than that obtained where *V. cholerae* and toxoid were administered.

M. Fluid Accumulation in Guinea-pig Ileal Loops in Response to Challenge with viable *V. cholerae*, after Immunisation with Toxoid or *V. cholerae* organisms, or Both

The vaccines tested in this study were not capable of eliciting marked protection of guinea-pigs from ileal-loop challenge with 2×10^6 viable *V. cholerae*. When the mean value of fluid accumulation was calculated for each group of four animals (Appendix 3), slightly reduced values (ml/cm) were noted in the following vaccine groups:-

(a) Three oral doses of toxoid alone in incomplete adjuvant, where ST208 was incorporated in the primary dose (GP 442-445, Appendix 3, Table 1).

(b) One i/m dose of toxoid in incomplete adjuvant containing ST208, followed by two oral doses of toxoid alone in incomplete adjuvant (GP 454-457, Appendix 3, Table 2).

Table 22. Complement-fixing antibacterial antibody response to immunisation with *V. cholerae* organisms alone or plus toxoid

Guinea-pig Code No. DST GP	Primary Immunisation	Reciprocal of CFT after:- 1 month no booster	2 months 1x0.3 ml oral booster after 6 weeks	3 months 2x0.3 ml oral boosters after 6 & 10 weeks
470-478	60 µg toxoid with $6x10^4$ *V.cholerae* in IA, oral capsule	16 16	16 16 16	32 8 16
491-499	100 µg toxoid with 10^5 *V.cholerae* in IA, i/m	8 16 8	32 32	32 32 32
512-520	60 µg toxoid with $6x10^4$ *V.cholerae* and 300 µg ST208 in IA, oral capsule	32 32 16	32 8	16 16 32
533-541	100 µg toxoid with 10^5 *V.cholerae* and 500 µg ST208 in IA, i/m	32 32	32 32	64 64 32
554-562	$6x10^4$ *V.cholerae* in IA, oral capsule	32 32 32	64 32 64	32 32 16
575-583	10^5 *V.cholerae* in IA, i/m	64 64	64 64	64 32
596-604	$6x10^4$ *V.cholerae* with 300 µg ST208 in IA, oral capsule	128 32 64	32 32	64 64 64
617-625	10^5 *V.cholerae* with 500 µg ST208 in IA, i/m	64 32	128 32 128	64 64 64

CFT = Complement fixation titre/0.025 ml serum.

IA = incomplete adjuvant: Octadecane, 2.5 ml; Bayol F, 2.5 ml; Arlacel, 1.0 ml; 1.9×10^6 *V. cholerae* in saline, 3.5 ml; or in 2.5 ml and toxoid, 1.0 ml.

(c) Three oral doses of toxoid + V. cholerae in incomplete adjuvant, where ST208 was incorporated in the primary dose (GP 508-511, Appendix 3, Table 3).

(d) One i/m dose of toxoid + V. cholerae in incomplete adjuvant containing ST208, followed by one oral booster dose of toxoid + V. cholerae in incomplete adjuvant (GP 525-528, Appendix 3, Table 4).

A student t-test was used to analyse the results obtained in Appendix 3. No highly significant decrease in "ml/cm" accumulated in test loops of the immunised animals, compared to unimmunised animals, was noted in this group of guinea-pigs. (Tables 23-28).

A typical ileal loop response to challenge with viable V. cholerae is shown in Plate 12.

Table 23. Statistical analysis of ileal loop test results - V. cholerae challenge

Analysis A. Based on difference in "ml/cm" values between challenge and control loops.

Analysis B. Based on difference in "ml/cm" values between immunised and unimmunised animals.

+ indicates significant protection from challenge.
- indicates no significant protection.

Guinea-pig Code No. DST GP	Vaccine	Route of administration	Time months	Challenge loop A	Challenge loop B	Control loop A	Control loop B
410 411 412 413	60 µg Toxoid in IA	oral x 1 0.3 ml	1	±	-	.	±
414 415 416 417	60 µg Toxoid in IA + booster after 6 weeks	oral x 2 0.3 ml	2	+	-	.	-
418 419 420 421	60 µg Toxoid in IA + boosters after 6 and 10 weeks	oral x 3 0.3 ml	3	±	-	.	-
434 435 436 437	60 µg Toxoid with 300 µg ST208 in IA	oral x 1 0.3 ml	1	+	-	.	-
438 439 440 441	60 µg Toxoid with 300 µg ST208 in IA + booster after 6 weeks, 60 µg Toxoid in IA	oral x 2 0.3 ml	2	+	±	.	-
442 443 444 445	60 µg Toxoid with 300 µg ST208 in IA + boosters after 6 & 10 weeks, 60 µg Toxoid in IA	oral x 3 0.3 ml	3	+	±	.	-
634-636 644-647	Non-immunised			±	.	.	.

See Appendix 3, Table 1 for raw data. See Appendix 4 for details of statistical analyses.

Table 24. Statistical analysis of ileal loop test results - V. cholerae challenge

Analysis A. Based on difference in "ml/cm" values between challenge and control loops

Analysis B. Based on difference in "ml/cm" values between immunised and unimmunised animals.

+ indicates significant protection from challenge
- indicates no significant protection

Guinea-pig Code No. DST GP	Vaccine	Route of administration	Time months	Challenge loop A	Challenge loop B	Control loop A	Control loop B
422 423 424 425	100 µg Toxoid in IA	i/m x 1 0.5 ml	1	+	-	.	-
426 427 428 429	100 µg Toxoid in IA + booster after 6 weeks, 60 µg Toxoid in IA	i/m x 1 0.5 ml; oral x 2 0.3 ml	2	+	-	.	±
430 431 432 433	100 µg Toxoid in IA + boosters after 6 & 10 weeks, 60 µg Toxoid in IA	i/m x 1 0.5 ml; oral x 2 0.3 ml	3	+	±	.	-
446 447 448 449	100 µg Toxoid with 500 µg ST208 in IA	i/m x 1 0.5 ml	1	+	-	.	-
450 451 452 453	100 g Toxoid with 500 g ST208 in IA + booster after 6 weeks, 60 g Toxoid in IA	i/m x 1 0.5 ml; oral x 1 0.3 ml	2	±	-	.	±
454 455 456 457	100 µg Toxoid with 500 µg ST208 in IA + boosters after 6 & 10 weeks; 60 µg Toxoid	i/m x 1 0.5 ml; oral x 2 0.3 ml	3	+	±	.	±

See Appendix 3, Table 2 for raw data. See Appendix 4 for details of statistical analyses.

Table 25. Statistical analysis of ileal loop test results - V. cholerae challenge

Analysis A. Based on difference in "ml/cm" values between challenge and control loops

Analysis B. Based on difference in "ml/cm" values between immunised and unimmunised animals.

+ indicates significant protection from challenge
- indicates no significant protection

Guinea-pig Code No. DST GP	Vaccine	Route of adminis-tration	Time months	Challenge loop A	Challenge loop B	Control loop A	Control loop B
458 459 460 461	60 µg Toxoid with 6 x 10^4 V. cholerae in IA	oral x 1 0.3 ml	1	+	-	-	-
462 463 464 465	60 µg Toxoid with 6 x 10^4 V. cholerae in IA + booster after 6 weeks	oral x 2 0.3 ml	2	+	-	.	-
466 467 468 469	60 µg Toxoid with 6 x 10^4 V. cholerae in IA + boosters after 6 & 10 weeks	oral x 3 0.3 ml	3	+	±	.	±
500 501 502 503	60 µg Toxoid + 6 x 10^4 V. cholerae with 300 µg ST208 in IA	oral x 1 0.3 ml	1	+	-	.	-
504 505 506 507	60 µg Toxoid + 6 x 10^4 V.cholerae with 300 µg ST208 in IA + booster after 6 weeks	oral x 2 0.3 ml	2	+	-	.	-
508 509 510 511	60 µg Toxoid + 6 x 10^4 V.cholerae with 300 µg ST208 in IA + boosters after 6 & 10 weeks	oral x 3 0.3 ml	3	+	±	.	-

See Appendix 3, Table 3 for raw data. See Appendix 4 for details of statistical analyses.

Table 26. Statistical analysis of ileal loop test results - *V. cholerae* challenge

Analysis A. Based on difference in "ml/cm" values between challenge and control loops

Analysis B. Based on difference in "ml/cm" values between immunised and unimmunised animals

\+ indicates significant protection from challenge
\- indicates no significant protection.

Guinea-pig Code No. DST GP	Vaccine	Route of administration	Time months	Challenge loop A	Challenge loop B	Control loop A	Control loop B
479 480 481 482	100 µg Toxoid with 1 x 10^5 *V.cholerae* in IA	i/m x 1 0.5 ml	1	+	±	.	-
483 484 485 486	100 µg Toxoid with 1 x 10^5 *V.cholerae* in IA + booster after 6 weeks	i/m x 1 0.5 ml; oral x 1 0.3 ml	2	±	-	.	±
487 488 489 490	100 µg Toxoid with 1 x 10^5 *V.cholerae* in IA + boosters after 6 & 10 weeks	i/m x 1 0.5 ml; oral x 2 0.3 ml	3	+	-	.	-
521 522 523 524	100 µg Toxoid + 1 x 10^5 *V.cholerae* with 500 µg ST208 in IA	i/m x 1 0.5 ml	1	+	-	.	-
525 526 527 528	100 µg Toxoid + 1 x 10^5 *V.cholerae* with 500 µg ST208 in IA + booster after 6 weeks	i/m x 1 0.5 ml; oral x 1 0.3 ml	2	+	±	.	-
529 530 531 532	100 µg Toxoid + 1 x 10^5 *V.cholerae* with 500 µg ST208 in IA + boosters after 6 & 10 weeks	i/m x 1 0.5 ml; oral x 1 0.3 ml	3	+	±	.	-

See Appendix 3, Table 4 for raw data. See Appendix 4 for details of statistical analyses.

Table 27. Statistical analysis of ileal loop test results - *V. cholerae* challenge

Analysis A. Based on difference in "ml/cm" values between challenge and control loops

Analysis B. Based on difference in "ml/cm" values between immunised and unimmunised animals.

\+ indicates significant protection from challenge
\- indicates no significant protection

Guinea-pig Code No. DST GP	Vaccine	Route of administration	Time months	Challenge loop A	Challenge loop B	Control loop A	Control loop B
542 543 544 545	6×10^4 *V. cholerae* in IA	oral x 1 0.3 ml	1	±	-	.	-
546 547 548 549	6×10^4 *V. cholerae* in IA + booster after 6 weeks	oral x 2 0.3 ml	2	±	-	.	±
550 551 552 553	6×10^4 *V. cholerae* in IA + boosters after 6 & 10 weeks	oral x 3 0.3 ml	3	±	-	.	±
584 585 586 587	6×10^4 *V. cholerae* with 300 µg ST208 in IA	oral x 1 0.3 ml	1	+	-	.	-
588 589 590 591	6×10^4 *V. cholerae* with 300 µg ST208 in IA + booster after 6 weeks	oral x 2 0.3 ml	2	+	-	.	-
592 593 594 595	6×10^4 *V. cholerae* with 300 µg ST208 in IA + boosters after 6 & 10 weeks	oral x 3 0.3 ml	3	+	±	.	±

See Appendix 3, Table 5 for raw data. See Appendix 4 for details of statistical analyses.

Table 28. Statistical analysis of ileal loop test results - *V. cholerae* challenge

Analysis A. Based on difference in "ml/cm" values between challenge and control loops

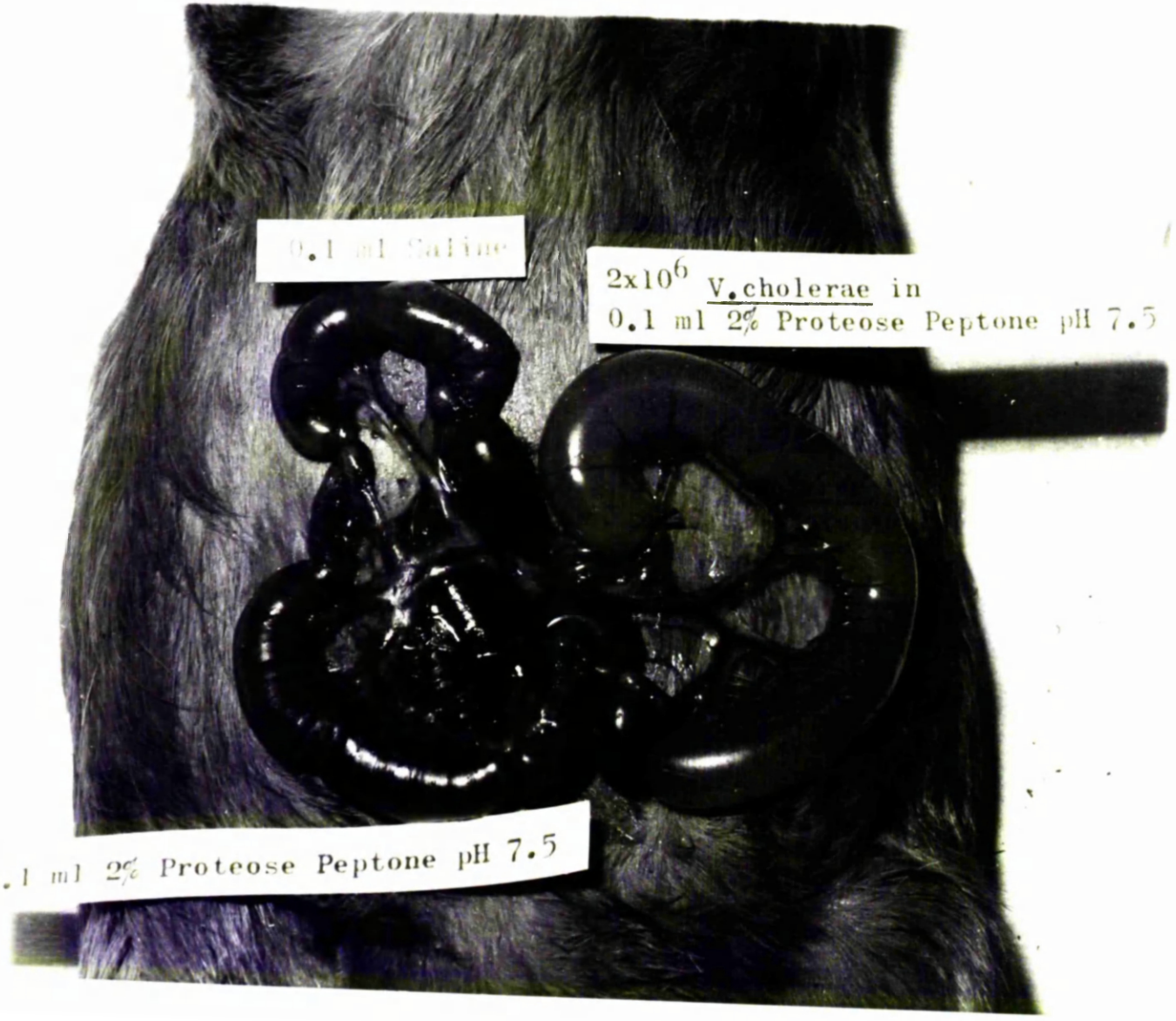
Analysis B. Based on difference in "ml/cm" values between immunised and unimmunised animals.

\+ indicates significant protection from challenge
\- indicates no significant protection

Guinea-pig Code No. DST GP	Vaccine	Route of administration	Time months	Challenge loop A	Challenge loop B	Control loop A	Control loop B
563 564 565 566	1 x 10^5 *V. cholerae* in IA	i/m x 1 0.5 ml	1	±	-	.	±
567 568 569 570	1 x 10^5 *V. cholerae* in IA + booster after 6 weeks	i/m x 1 0.5 ml; oral x 1 0.3 ml	2	±	-	.	±
571 572 573 574	1 x 10^5 *V. cholerae* in IA + boosters after 6 & 10 weeks	i/m x 1 0.5 ml; oral x 2 0.3 ml	3	+	-	.	-
605 606 607 608	1 x 10^5 *V. cholerae* with 500 µg ST208 in IA	i/m x 1 0.5 ml	1	+	±	.	-
609 610 611 612	1 x 10^5 *V. cholerae* with 500 µg ST208 in IA + booster after 6 weeks	i/m x 1 0.5 ml; oral x 1 0.3 ml	2	+	-	.	-
613 614 615 616	1 x 10^5 *V. cholerae* with 500 µg ST208 in IA + boosters after 6 & 10 weeks	i/m x 1 0.5 ml; oral x 2 0.3 ml	3	+	-	.	-

See Appendix 3, Table 6 for raw data. See Appendix 4 for details of statistical analyses.

Plate 12. Fluid accumulation in guinea-pig ileal loops sixteen hours after injection of viable *V. cholerae*.


0.1 ml Saline
2x10^6 V.cholerae in
0.1 ml 2% Proteose Peptone pH 7.5
0.1 ml 2% Proteose Peptone pH 7.5

DISCUSSION

1. Purification of Crude Enterotoxin

One of the problems in the purification of cholera enterotoxin is the spontaneous conversion of toxin to toxoid (Finkelstein et al, 1971b). Mosley et al (1970a) recommended borate gelatin saline (B.G.S.) for making toxin dilutions in the i/d blueing test as the gelatin acts as a protective colloid (see p. 29).

It was decided to use the B.G.S. of Mosley et al in gel filtration procedures. A combination of Sephadex G-100 and G-200 filtration provided a toxin-containing peak. Crude culture medium products were removed as a brown band on G-100, Method A (p. 104). A similar result was obtained when G-100 and G-200 columns were run in series, Method B (p. 107).

It was noted in these studies that the toxin was eluted directly following the void column volume. This was unexpected with enterotoxin of 84k daltons, particularly with Sephadex G-200 which has a retention range up to 200k daltons. It was concluded that this effect was due to the use of B.G.S., containing gelatin, as eluant. A decrease in the effective exclusion limit was possibly caused by the presence of gelatin within the Sephadex beads, thus reducing the pore size.

Consequently, it was apparent that an effective toxin-containing preparation was obtained using a one-step filtration procedure on G-200 with B.G.S. as eluent (see Fig 8). The toxin-containing fractions eluted from the G-200 column (method C, p. 107) were pooled and tested in the rabbit i/d blueing test, whereupon it was found that 2 μg of the protein contained in this peak represented 1 B.D. This was in comparison to the crude (NIH) preparation used as the starting material,

4 μg of which equalled 1 B.D. This result was encouraging in view of the observation by Duhamel et al (1970) that an increase in specific activity did not accompany purification of cholera toxin, because of the spontaneous loss of activity during the purification process.

Using method C, there was little difference between the elution profiles obtained with the crude NIH toxin and the culture filtrate of V. cholerae NCTC 7254. As described previously this organism was used throughout this study.

As far as can be ascertained no other workers have used BGS as eluant in purification procedures and it would seem that this simple one-step technique provides enterotoxin preparations which retain biological activity.

2. Biological Tests in Cholera Research

It became apparent during this study that there were numerous factors which had to be taken into consideration in biological tests.

A. Intra-dermal Blueing

The toxoid was prepared by treatment of V. cholerae culture filtrate with formalin. Toxoid prepared by this method was noted by Finkelstein (1976) to be unstable and to revert to low levels of toxicity. The observation that the Wellcome toxoid contained 40 B.D./ml was not unexpected, however, it indicates the need to consider the presence of active toxin in vaccines, since it is known that active toxin can profoundly influence the immune response (see p. 184).

In these studies the blueing activity of the NIH crude toxin was measured and it was shown that 4 μg ≡ 1 B.D. The data from the National Institute of Health indicated that 5 μg ≡ 1 B.D.; the slight difference was probably due either to the prolonged storage of the

freeze-dried toxin or to the fact that rabbits and not guinea-pigs were used.

In one instance during an i/d blueing inhibition test no areas of blueing were found on the rabbit skin, although eighty different injections, including eight containing active toxin, were administered. There seemed to be no explanation for the lack of response in this animal, other than the marked inter-animal variation in sensitivity to i/d injection of cholera toxin (Craig, 1968, see p. 51). Craig concluded that a titration of toxin of known potency should be included in each animal. This conclusion is endorsed by the discovery of a non-responder rabbit in the present work.

Initial experiments showed clear-cut areas of blueing in the skin of hairless, hrhr mice. The injection of 4 μg NIH toxin (1 B.D. as determined in rabbit i/d blueing tests) was also found to result in a 7 mm diameter area of blueing on the inner surface of the skin of hairless mice. Unfortunately, because of the necessity to include a titration of active toxin in each animal it was impracticable to use mice routinely. The test proved to be useful, however, for detecting enterotoxic activity associated with peak fractions from Sephadex columns.

B. Ligated Ileal Loop

Several variables associated with the ileal loop test influence the measurement of fluid accumulation, and hence toxin neutralisation in vivo. (a) The convoluted surface of the intestine makes accurate measurement of a 5.0 cm loop difficult. (b) After injection of toxin the muscular layers of the intestine stretch to allow fluid accumulation. This process is limited by a maximum stretch value, which

causes the plateau effect shown with high doses of toxin (Fig 11). A reduction in fluid accumulated (∿5.0 ml fluid/loop or ∿0.7 ml fluid/cm loop, found on the upper plateau in loops containing 250-1000 μg enterotoxin) is indicative of considerable toxin-neutralising activity in the intestine. Conversely, the neutralising activity against low doses of toxin (5-25 μg) is not possible to detect. For these reasons it was decided to use the higher dose levels and test for complete neutralisation.

In one non-immunised guinea-pig, weighing ∿ 250 g, purchased from Porcellus Animal Breeding Ltd., ileal loops injected with 250 μg, 500 μg and 1 mg of enterotoxin contained considerably less fluid than accumulated in similar loops in adult non-immunised guinea-pigs. Although no similar studies have been performed in guinea-pigs, one of the factors considered in the standardisation of the rabbit ileal loop test (see p. 49) was the age of the animal. All other ileal loop tests in this work were carried out in adult guinea-pigs, and to reduce other interanimal variables each test, including the toxin titration, was carried out in several animals.

Ileal loop results are generally presented as a value of ml of fluid accumulated per cm of loop (see p. 49). However, it is necessary to point out the limitations of this practice. An identical "ml/cm" result may be obtained for loops which differ greatly in the volume of fluid accumulated, e.g. 10 ml/10 cm = 1.0 and 5 ml/5 cm = 1.0. There are two possibilities which explain why the latter loop contains 5 ml as opposed to 10 ml:- (a) the initial length of loop tied off (∿5 cm) did not stretch significantly beyond 5 cm and could only accommodate a maximum of 5.0 ml fluid. If this volume was the maximum which could accumulate in a 5.0 cm loop the practice of presenting results as a ratio

is valid; (b) conversely, the animal may have been immunised and antibodies present in the gut prevented maximum fluid accumulation. In this case, the 5.0 ml of accumulated fluid may be insufficient to stretch the loop beyond the 5 cm originally ligated. Thus, the reduction in fluid accumulation could be obscured by presenting results as the ratio of ml/cm.

For these reasons the results of ml fluid per loop were also considered. However, data in this form are also questionable. Due to the convolutions of the gut and contractions of the muscular wall it is impossible to measure an exact 5.0 cm loop. Where maximum fluid accumulation occurs the volume of fluid is controlled, at least partly, by the length of ligated loop, as mentioned above. Evidence from control experiments suggests that the volume of accumulated fluid may depend on the particular segment of gut used. Loops (5.0 cm) were distended by injecting saline, and were found to rupture after injection of 4.0-7.0 ml. Although the stretching time in control experiments was accelerated it would seem that the element of variation among results expressed as ml of fluid is probably high. This conclusion was supported when the coefficients of variation of "ml" and "ml/cm" values were considered (Fig 13). The results of ileal loop tests were therefore presented in the accepted form of volume/length.

An assessment of the protective activity of a particular vaccine was by consideration of "ml/cm" results and the results of statistical analyses A and B (Appendix 4). It became apparent that where the raw data indicated a decrease in fluid accumulation this was confirmed by the t-test analysis B (where the decrease in fluid accumulation between immunised and unimmunised animals was considered).

It is generally accepted, now, that the intestinal fluid accumulation caused by cholera enterotoxin is the result of increased levels of cAMP in mucosal cells (see p. 37). However, Cedgård et al (1978) presented evidence showing that cholera toxin caused a 50% increase in blood flow in feline small intestine after exposure of the intestinal mucosa to enterotoxin. Despite the observed doubling of mean blood flow in the mucosa-submucosa, no change in blood flow in muscle tissue was noted. These workers suggested that such haemodynamic changes in the intestine may be at least partly involved in the pathogenesis of cholera.

In the present study it was noted that where guinea-pigs were protected from ileal loop challenge by immunisation, the volume of fluid accumulating in "control" loops injected with BGS, was less than the volume in non-protected animals. This observation was difficult to explain in terms of the cAMP mechanism since (a) leakage of the toxin from test to control loops was unlikely ~~due to the presence of~~ because there was an interloop and (b) cholera toxin acts on cells to which it is bound, i.e. there is no known lateral transmission of adenyl cyclase activation along the intestine.

However, the observation becomes explicable if, as suggested by Cedgård et al (1978), cholera toxin can induce increased blood flow to the whole intestine and cause a general efflux of fluid. In this case immunisation would presumably decrease the toxic effect and therefore prevent, to some extent, the increase in blood flow.

If this is so, protection against ileal loop challenge with enterotoxin in immunised guinea-pigs is probably best indicated by t-test analysis B, where the 4 loops in the immunised animals are compared to the 4 loops in the non-immunised group.

Consideration of enterotoxin-challenged loops by t-test analysis A indicates groups of guinea-pigs in which the test loops do not contain significantly more fluid than the control loops injected with B.G.S. This is indicative of complete neutralisation of enterotoxin by groups of immunised guinea-pigs, only where analysis B has also indicated protection (e.g. GP386-397, Table 20). Obviously where analysis B did not indicate protection the result of analysis A may have been affected by the effect suggested by Cedgård et al (1978).

3. Immunisation of Mice with Cholera Vaccines

A variety of experiments was done in mice, using both toxoid and V. cholerae organisms in vaccines, in an attempt to determine the most suitable combination of adjuvant and antigen concentrations to add to vaccines.

There is substantial evidence that serum antibodies raised against cholera antigens are capable of eliciting protection against the disease. For example, Burrows and Kaur (1975) noted that high levels of serum antitoxin were found during convalescence or in asymptomatic cholera infections, which indicated the importance of these antibodies in human beings. In rabbits Svennerholm and Holmgren (1977) found that s/c immunisation with V. cholerae LPS and enterotoxin gave rise to a serum and gut-associated antibody response, and resulted in greater levels of protection than enteral immunisation, which gave rise only to a gut-associated response. This suggested a significant contribution of serum antibodies to protection against intestinal challenge with V. cholerae or its enterotoxin. Also in a series of studies, summarised by Pierce, Sack and Sircar (1977), a correlation was found repeatedly between serum antitoxin and levels of protection against cholera infection in dogs.

A. Effect of Adjuvant

The present work demonstrated that it is possible to stimulate the serum antibody response to cholera toxoid using whole mycobacterial cells or water-soluble glycopeptide. Consideration of Fig 12 shows that 15-60 μg of M. tuberculosis whole organisms and 10-40 μg toxoid, or with 15-60 μg of mycobacterial glycopeptide, ST208, and 1.25-40 μg toxoid stimulated antitoxin production. It is notable that water-soluble glycopeptide was more adjuvant-active, on a weight basis, than whole mycobacterial cells, and stimulated an antitoxin response to 1.25 μg of cholera toxoid (Table 7).

B. Comparison of NIH Toxin and Toxoid

The apparent difference in immunogenicity of cholera toxin (NIH) and cholera toxoid may be explained by their composition. The NIH crude enterotoxin contains a variety of vibrio cell products and culture medium constituents, as well as cholera toxin. Thus although 80 μg and 160 μg of this material was injected i/p per mouse, the actual dose of toxin antigen would be considerably lower. The toxoid preparation was immunopurified, so the amount of this material injected would correspond to the toxoid antigen dose per mouse.

It was reported that native cholera toxin was more immunogenic than its toxoid (Holmgren et al, 1972) and the method of toxoiding may affect the antigenicity of the molecule (Holmgren and Svennerholm, 1977; Pierce et al, 1977). In the present work as a crude toxin preparation was used it is not possible to comment on the antigenicity, and the difference is attributed to purity of the two preparations.

Verwey et al (1975) suggested that less purified preparations of cholera toxin were capable of eliciting higher levels of antitoxin

than purified forms, possibly through the adjuvant effect of contaminating LPS. No such effect was found in the present work and Rappaport et al (1976) believed that small amounts of somatic antigen did not exert an adjuvant effect on cholera toxoid.

C. Composition of Vaccines

It has been suggested (Finkelstein, 1976; Germanier et al, 1977; Holmgren et al, 1977a; Svennerholm, and Holmgren, 1977) that it may be advantageous to use both V. cholerae somatic and toxin antigens in a vaccine. However, measurable quantities of V. cholerae LPS may be able to exert an adjuvant effect on cholera toxin (Verwey et al, 1975; Germanier et al, 1977) and cholera toxin is certainly adjuvant-active in some cases (Lindholm et al, 1977). It is therefore of importance to know whether or not the presence of LPS in a vaccine affects the immune response to enterotoxin and vice versa.

From the results obtained for A.U./ml of serum from mice immunised with combined vaccines, the presence of V. cholerae did not appear to influence the antitoxic immune response to any extent, either in saline or incomplete adjuvant. The low PHA titres obtained are difficult to account for since both the i/d blueing inhibition test and the PHA test monitor levels of the same antibody. The discrepancy in results is at least partly accounted for by the limitations of the PHA test (see p.193).

The toxoid used in these studies was found to revert to low levels of toxicity (see p. 111) and therefore may have been capable of increasing the immune response to V. cholerae when injected simultaneously with the organisms. However this was not indicated by the complement fixation titres obtained. The lower titres obtained in some

cases may be an indication that a low level of toxoid present in the vaccine preferentially competes with the somatic antigens of V. cholerae. The exception to this was found with 40 μg toxoid plus 1 x 10^6 V. cholerae organisms in saline. This effect appears to be overcome by the incorporation of the two antigens in incomplete adjuvant (see Table 10).

4. Immunisation of Guinea-pigs with Cholera Vaccines

A. Mineral Oil Component of Vaccines

At the commencement of this study it was decided to use innocuous "liquid paraffin, refined for medicinal use as emollient" as the oil in water-in-oil emulsions. A preliminary study was carried out to investigate the possibility of immunising guinea-pigs by oral administration of cholera toxoid. The results obtained with liquid paraffin in the suspending medium were generally disappointing. It was not possible to elicit a serum antibody response by purely oral immunisation. No conclusions could therefore be drawn about the relative advantage of using whole mycobacteria or water-soluble glycopeptide, or about the incorporation of liquid paraffin into the adjuvant mixture.

Subsequently, Stewart-Tull et al (1976) showed that the length of the carbon chain of the hydrocarbon component of mineral oils was important to the stimulation of the immune response by adjuvants. Subsequently, all experiments were done using Bayol F preparations, known to contain predominantly long chain hydrocarbons ($nC_{15}H_{32}$-$nC_{20}H_{42}$), and octadecane ($CH_3(CH_2)_{16}CH_3$).

B. Mycobacterial Component of Vaccines

It was possible to stimulate the immune response in guinea-pigs

to cholera toxoid, by incorporating either whole mycobacteria or water-soluble glycopeptide preparations into water-in-oil emulsions (Table 12, GP Nos. 280-301). In one instance, i.e. with ST210, poor results were obtained and this was attributed to slight variations in the glycopeptide-preparation procedure.

With Freund's complete adjuvant the variability in response within the group was partly accounted for by the difficulty in obtaining an even suspension of organisms in the vaccine mixture. Thus each animal did not necessarily receive the same amount of adjuvant. However, with the glycopeptide preparations, the animals received more closely equalised inocula as the material was in the form of a proper solution (Stewart-Tull et al, 1975). The observed differences in response in the latter groups of animals are attributed to interanimal variation.

C. Route of Administration of Toxoid Vaccines (G.P.303-320)

The observation in this study that it is not possible to stimulate serum antibody production by oral immunisation, has been corroborated by the work of Svennerholm and Holmgren (1977). After immunisation of rabbits with cholera antigens (V. cholerae LPS and enterotoxin) they monitored the production of specific antibodies in different tissues and related the response to the route of immunisation. Enteral immunisation stimulated antibody formation in intestinal tissue and Peyer's patches but not in the spleen and no serum antibodies were detected.

Guinea-pigs 318 and 319 which were given simultaneous oral and i/m primary inocula of toxoid in incomplete adjuvant containing glycopeptide ST208 followed by one oral booster dose, gave precipitin

lines in the Ouchterlony test. The results of A.U./ml obtained for this group of guinea-pigs (Table 13) indicated that there was no distinct advantage in giving the dual primary immunisation. The gel diffusion results (Table 18) may be an indication that this conclusion is not completely correct since no precipitin line was obtained where animals were given separate oral or i/m primary doses of this particular vaccine (i.e. G.P. Nos 308-311 and 312-314 respectively).

The results in Table 13 did not allow conclusions to be drawn about gut-associated antibody production in response to i/m immunisation alone, since all animals received a subsequent oral booster dose. Svennerholm and Holmgren (1977), however, found parenteral immunisation (s/c) to induce antibody production in the spleen and intestinal tissue. Similarly the recent results of Svennerholm et al (1977) suggest that parenteral immunisation may result in a secretory antibody response at mucosal surfaces.

Although a correlation exists between serum antibody titres and levels of protection against cholera (Burrows and Kaur, 1975; Holmgren and Svennerholm, 1977; Pierce et al, 1977), a similar observation for gut-associated antibody levels has not been reported. Pierce and Sack (1977) found protection against virulent challenge in dogs, immunised by a parenteral-oral sequence, after antitoxin levels in intestinal washings had declined. These workers suggested that intestinal immunity could best be measured by challenge experiments. Pierce and Reynolds (1975) showed that the jejunal secretory immunological system of dogs apparently possessed immunological memory, and a rapid secondary antibody response to locally administered antigen could occur. It is therefore of significance to note that in the present investigation an intestinal antibody response was obtained,

although at a low level. Further experiments would be required to determine whether the stimulation of this gut-associated antibody response was an indication that immunological memory in the gut had been established. Unfortunately such experiments, in which the time between the primary and oral booster dose administration would be extended, were not possible within the time scale of the present investigation.

The method used here to measure gut-associated antibody was similar to that employed by Kaur et al (1971). The luminal contents were removed from the intestine since Aziz et al (1968) had shown both faeces and succus entericus to nullify enterotoxin activity, which would have interfered with the i/d blueing inhibition test. This crude method of obtaining gut-associated antibody does not, therefore, isolate secretory antibody present in the lumen of the gut, and probably does not obtain all of the antibody associated with mucosal cells. In addition, no account is taken of antibody degradation by proteolytic enzymes which may be present in these preparations. That antibodies are isolated at all by this method is verified in Plate 10, where the gut tissue extracts were tested by Ouchterlony gel diffusion against anti-guinea-pig globulin antiserum. The method is useful, therefore, to detect the presence of an intestinal antibody response (gut-tissue extracts from six unimmunised guinea-pigs were found to contain less than 0.1 A.U./ml) and not to quantitate the extent of this response accurately.

Lastly, it was observed by Kaur et al (1971) that variability in the levels of antitoxin in gut homogenates was reduced on precipitation with 50% $(NH_4)_2SO_4$, after which consistent results were obtained. This conclusion was disputed in this study, since all homogenates were

precipitated before testing. Replicate tests of the protein solutions obtained did not necessarily yield identical results of antiblueing activity. It is realised that thawing the preparations from -20°C may have allowed proteolysis of antibody, since some results varied between the initial test and subsequent tests at later dates.

D. Frequency of Administration of Toxoid Vaccines (GP 333-406)

Some protection against fluid accumulation in the ileal loop test could be obtained by a purely oral immunisation schedule (Fig 14). However the protective response in guinea-pigs given i/m primary doses of toxoid in both incomplete adjuvant alone or incomplete adjuvant plus ST208, was superior to the response obtained with oral priming with incomplete adjuvant alone or plus ST208, respectively (Appendix 2, Tables 1 and 2).

The finding that i/m priming elicits a greater protective response was corroborated by Pierce and colleagues (Pierce and Reynolds, 1975; Pierce, 1976; Pierce and Sack, 1977; Pierce et al, 1977) who consistently observed that parenteral priming with cholera toxoid, in rats and dogs, induced a greater response in the intestine after oral boosting, than did oral priming followed by oral boosting. It was suggested that protection in dogs, immunised by a parenteral-oral sequence, resulted from secretory antibody elaborated in the lamina propria of the gut. This conclusion was reached since protection, against intrajejunal challenge with V. cholerae or its enterotoxin, was evident after the serum antibody response to the parenteral primary dose had diminished.

In this study the serum antitoxin levels induced by i/m priming did not greatly diminish over the three month period of the experiment.

It is impossible therefore to state that antibody derived from the serum was not responsible, at least in part, for the protection observed. Alternatively, where a purely oral schedule was employed, protection of the guinea-pigs presumably did result from an antibody response at the surface of the gut, since serum antitoxin titres were not detected (GP nos 333-343 and 356-364, Table 14).

That detectable levels of immunity to enterotoxin challenge in the ileal loop were stimulated by two oral immunisations is of note. It has been observed previously that oral immunisation requires repeated administration of antigen to produce any protective effect (Pierce and Sack, 1977). For example, Pierce et al (1977) found attempts to immunise dogs by the oral route to be "strikingly ineffective."

The relative success of the oral immunisation scheme used here may result from the administration of toxoid in a water-in-oil emulsion enclosed in a gelatin capsule. Previously, fluid forms of toxoid were used in attempts to stimulate gut-associated antibody responses. For example, rats were either "immunised" orally, by replacing their drinking water with a toxoid solution, or intra-intestinally by injecting toxoid, in fluid form, directly into the lumen of the gut (Pierce and Gowans, 1975). Craig and Cebra (1973) suggested that the difficulty encountered in stimulating an immune response in the intestine could be explained if gut-associated lymphoid tissue lacked or had a less efficient antigen trapping system than other types of lymphoid tissue. Stimulation of gut-associated immune mechanisms is also further complicated by the enzymic degradation of the immunising antigen and its constant removal from the intestine by peristalsis.

The use of a water-in-oil emulsion possibly overcomes the problem of expulsion of the antigen from the gut. This material is

relatively "sticky", and in some guinea-pigs, could be seen to have adhered to the intraluminal surface. This adherence would presumably retard the removal of antigen and localize it in the gut where it may interact with gut-associated lymphoid tissue.

The incorporation of the oral vaccine in a gelatin capsule may have helped to overcome a second major problem with oral immunisation, namely the effect of gastric acidity. The denaturation of proteins by acid obviously greatly affects their antigenicity and is therefore undesirable. Where the vaccine was contained in a capsule, the capsule itself would have to be destroyed before the vaccine could be attacked. Thus the use of these capsules may prevent or at least decrease the exposure of antigen to gastric acidity. Similarly, the water-in-oil emulsion may provide some protection for the toxoid against the proteolytic enzymes in the small intestine.

In guinea-pigs where i/m priming was used, glycopeptide ST208 was obviously adjuvant-active, both in terms of serum antitoxin production (Table 14, GP nos 398-406) and induction of levels of protection against ileal loop challenge with enterotoxin (Table 20, GP nos 386-397). Protection, measured as a decrease in fluid/cm of ileal loop, was apparent in these guinea-pigs against all challenge doses of toxin.

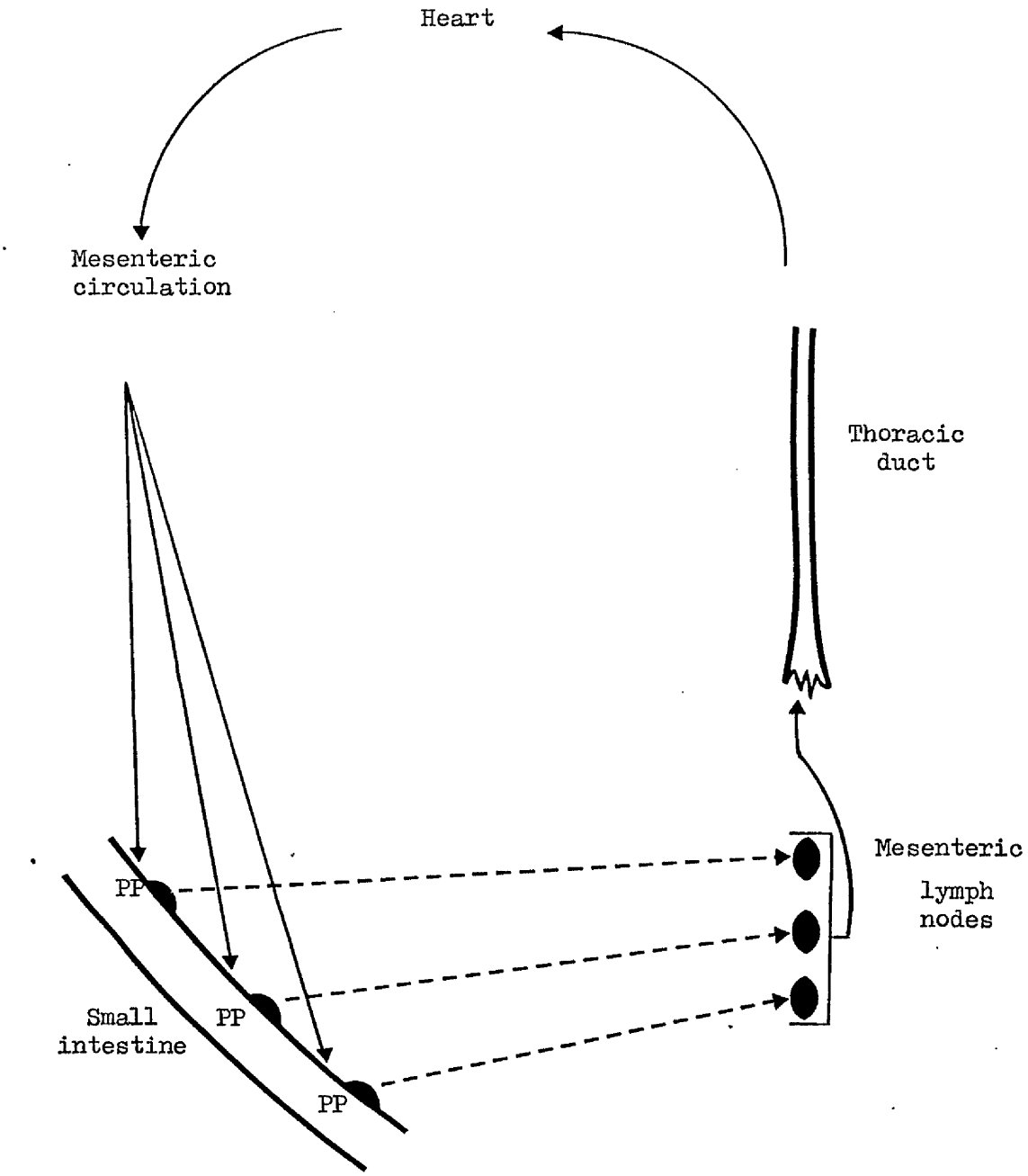
When starting this work it was considered worthwhile to investigate mycobacterial adjuvants in oral immunisation. This preliminary study showed no distinct advantage in incorporating mycobacterial glycopeptide ST208 in oral vaccines (Table 19). Nevertheless better results might emerge if a broad range of adjuvant concentrations was studied. Pierce and Sack (1977) drew attention to the lack of knowledge

about substances which are adjuvant-active at mucosal surfaces. They pointed out that if such substances exist, it might be possible to simplify oral immunisation schedules and also reduce the dosage of antigen. However it must be borne in mind that such adjuvant substances may also have the capacity to induce hypersensitivity to other antigens present in the gut, e.g. food antigens. If this were so, the use of these substances would be unacceptable.

It is accepted that a gut-associated immune response is due to the production of IgA at the mucosal surface (Craig and Cebra, 1973; Guy-Grand, Griscelli and Vassalli, 1975). A mechanism whereby antigenic stimulation at the mucosal surface can result in this IgA response has gradually emerged (Marsh, 1975; Solomon and Taub, 1976; Hall, Hopkins and Orlans, 1977; Beh, 1977; McWilliams, Phillips-Quagliata and Lamm, 1977). Although Marsh (1975) concluded that after antigenic stimulation, maturation of small lymphocytes to antibody secreting plasma cells occurred within the interepithelial cell spaces, the other groups were in favour of maturation occurring at a site other than the gut. Stimulated immunocytes were thought to migrate from Peyer's patches, following the path shown in Fig 16, before "homing" back to the small intestine. Maturation of the lymphocytes was thought to occur at some point during this migration, probably in the mesenteric lymph nodes (McWilliams et al, 1977) so that on return to the lamina propria of the gut, via the blood stream, they are capable of antibody secretion.

Since cholera is prevalent in the developing countries, the advantages of having a simple method of immunising large sections of the community are obvious. For example, the requirement for highly skilled personnel to administer the vaccine would be abolished if an oral

Figure 25. The proposed route of migration of gut-associated small lymphocytes



PP = Peyer's patches

immunisation programme could be employed. One of the major problems found with cholera immunisation in human beings has been the limited duration of protection from infection (see p.70). However, encouraging results (Pierce, 1976) have been obtained which suggest that using oral immunisation after initial parenteral priming may lengthen the duration of protection. Pierce suggested that the parenteral-oral immunisation sequence may elicit a very rapid secondary response in the gut to the antigen, with the interruption of the disease process within its incubation period. He considered that if dogs could be protected against a viable challenge of approximately 1×10^{11} V. cholerae within a 12 hr incubation period by such a secondary response, it was not unlikely that human beings could similarly be protected from infection, where the infecting dose may well be lower, and where the average incubation period is 50 hr.

In the present study, regardless of i/m or oral priming, the protective response induced was superior after one than after two oral booster doses (Figs 14 and 15). This response is similar to that noted by Pierce et al (1977) who found that as the time between the administration of the s/c primary injection and the oral booster dose was increased, the mucosal immune response to cholera toxoid was reduced rather than stimulated. The toxoid preparation used in this work, as in much of Pierce's work, is a formal toxoid which was shown, in the rabbit i/d blueing test, to revert to low levels of toxicity (see p. 111). It is well documented that active cholera toxin may act as an adjuvant, probably through its effect on small lymphocytes (Northrup and Fauci, 1972; Chisari and Northrup, 1974; Chisari, Northrup and Chen, 1974; Holmgren, Lindholm and Lönnroth, 1974; Kateley and Friedman, 1975; Douglas, Zuckerman and Ooka, 1976; Lönnroth and Lönnroth, 1977). The

resultant immune response is dependent on the time at which cholera toxin is administered in relation to the antigen (Holmgren and Lindholm, 1976; Lindholm et al, 1977). It was found that 1 μg of cholera toxin stimulated IgM and IgG antibody formation to sheep erythrocytes when the toxin was given simultaneously with the antigen. Given two days after the antigen the toxin caused a suppression of the antibody response. A further injection of cholera toxin two or three days before secondary antigen injection decreased the anamnestic IgG response; given simultaneously there was an increase in the number of antibody producing cells.

Is it possible that repeated administrations of cholera toxoid, known to revert to toxin (p. 111), causes suppression of the immune response to the toxoid in a similar manner to the suppression of the immune response to sheep red blood cells by cholera toxin?

An alternative to this conclusion was suggested by Svennerholm et al (1977) who thought it possible that the difficulty in boosting mucosal responses by local administration of antigen, was due to pre-existing antibodies in the gut preventing contact between the immunising antigen and the submucosal lymphoid tissue. If this is correct the second oral toxoid booster used in this work would be incapable of producing a stimulatory effect due to the presence of antitoxin in the intestine as a result of the primary inoculation and the first oral booster. The protection which was evident at the third month challenge date, where an i/m injection of toxoid in incomplete adjuvant containing glycopeptide ST208 was the primary dose (Table 20, GP nos 394-397), would probably be due to the immune response to the primary and secondary doses, which would have fallen off slightly by this time.

Craig and Cebra (1973) suggested that IgA antibody synthesis in the gut may contribute to circulating IgA, however no evidence of this was obtained (Table 14) even after two oral immunisations which stimulated some levels of antibody formation in the gut, as judged from protection studies (Fig 14). The serum antitoxin response elicited by i/m priming did not decline over the duration of three months (Table 14). Since two or three oral doses did not result in detectable serum antitoxin levels, it is considered unlikely that the serum antibody titre stimulated by i/m injection was maintained by an additive effect of oral boosting.

Sera from guinea-pigs which received an i/m primary dose of toxoid in incomplete adjuvant alone or supplemented with glycopeptide ST208 (GP nos 377-385 and 398-406) showed lines of precipitation in Ouchterlony gel diffusion tests. No precipitin lines occurred with sera from animals immunised purely by the oral route (Table 18).

No correlation existed between the serum antitoxin titres obtained above and levels of protection against ileal loop challenge, where similar vaccination schedules were used. This was expected where purely oral immunisation was used since levels of protection were probably due to IgA production in the intestine. Where i/m priming was used some contribution of serum antibody to the protective response may have occurred. It was noteworthy that a single i/m dose of toxoid in incomplete adjuvant + ST208 elicited protection against ileal loop challenge with enterotoxin, whereas i/m injection of toxoid in incomplete adjuvant alone did not (Fig 15). In contrast, similar serum antitoxin responses were produced by both vaccines (Table 14).

The latter point raises the question of the effect of mycobacterial glycopeptide ST208 on the intestinal immune response. In

general, researchers are reluctant to assume that the serum antibody response to immunisation reflects the immune status of the gut. For example, Rappaport et al (1976), concluded that low levels of somatic antigen (LPS) did not exert an adjuvant effect with respect to serum antibody production against cholera toxoid. However, they did not rule out the possibility that such levels of somatic antigen may have some effect on intestinal antitoxin responses to parenteral immunisation with toxoid. From the results discussed above it seems possible that glycopeptide ST208 is capable of producing a stimulatory effect on intestinal immune mechanisms, after parenteral administration (GP nos 386-389, Appendix 2, Table 2) which is not evident from serum antibody titres (Table 14).

Since i/m priming resulted in greater protection against enterotoxin challenge in ileal loops than did oral priming, it is impossible to dismiss the potential contribution of serum antibodies to the protective response. However the difference in response following i/m and oral priming may ~~also be due to~~ support the suggestion that i/m priming induces a greater level of immunological memory in the gut than does oral priming (Pierce and Gowans, 1975; Pierce and Reynolds, 1975; Pierce and Sack, 1977; Pierce et al, 1977).

The levels of protection in guinea-pig nos 321-406 bore no relation to the gut-associated antibody responses observed (Table 15), where similar immunisation schedules were used. Also results obtained with guinea-pig nos 303-320 (Table 13) for gut-associated antibody, showed no correlation with the results from these animals. Previously, responses were evident after i/m plus one oral administration; however no response was apparent in guinea-pigs 377-385 or 398-406, using

similar vaccines. In addition, with guinea pigs 303-305 and 309-311 (Table 13) gut-associated antitoxin levels were evident after two oral doses of toxoid, while, in Table 15, gut-associated antitoxin levels were not detectable after one, two or three oral toxoid doses. It is difficult to reconcile the differences in these two sets of results. One possible explanation is that gut extracts were tested five months after preparation for GP 303-320, and eight months after preparation, for the group 333-406. The tissue-extracts were stored at $-20^{\circ}C$ during this time, and the natural stability of the preparations may have been affected.

E. Incorporation of V. cholerae Organisms into Vaccines (GP 410-616)

The fluid volumes obtained with ileal loop challenges with V. cholerae were generally greater than those obtained with enterotoxin challenge. This effect was thought to be due to the osmotic activity of peptone water in which the vibrios were injected. Control loops injected with BGS contained less fluid than control loops injected with peptone water, presumably because BGS did not cause such an influx of water into the intestinal lumen. It should be noted that 0.1 ml volumes of 2% proteose peptone containing 2×10^6 V. cholerae organisms, used for virulent challenge, did not contain detectable enterotoxin.

Unfortunately, due to the high levels of fluid accumulation induced by injection of peptone water, it was impossible to draw any conclusions from analysis A (Appendix 4) t-test results. In several groups of animals there was no significant difference between "ml/cm" in challenge and control loops (Tables 23-28), which normally might have indicated protection. However from the raw "ml/cm" data (Appendix 3)

it is obvious that these guinea-pigs were not protected. Statistical assessment of these results was therefore dependent on analysis B (Appendix 4), which, it is realised, may also have been affected by the influence of the osmotic activity of peptone water. Protection against V. cholerae challenge may not have been obvious due to the small differences in fluid accumulation in loops receiving V. cholerae in peptone water and those receiving only peptone water (c.f. Appendix 3, Table 7).

No highly significant reduction in fluid accumulation between immunised and non-immunised guinea-pigs was found in any of the groups of animals challenged with viable V. cholerae (Tables 23-28).

The immunising dose of V. cholerae used in this series of experiments was 1×10^5 organisms per animal with i/m injection, and 6×10^4 organisms with oral administration (Appendix 3, Tables 3-6). The immune response in human beings to cholera vaccination is known to be dose dependent, the maximum dose (approx. 1×10^9) which can be administered is limited by the inherent endotoxic properties of the organisms (see p. 70). In order to avoid adverse reactions to the vaccine in guinea-pigs, lower numbers of organisms were chosen; however it appears that the dose employed may have been insufficient to stimulate a significant response. A higher dose of organisms might have improved the immune response.

It was disappointing that vaccination schedules which were shown to protect guinea-pigs against ileal challenge with enterotoxin (Table 20, GP 386-397) did not elicit detectable protection against challenge with viable V. cholerae (Table 24, SP 446-457). Apart from the difficulties mentioned above, in distinguishing protection in these studies, there are several possible explanations for the lack of

protection elicited by these toxoid vaccines against V. cholerae challenge. First, with enterotoxin challenge, these vaccines were shown to elicit protection against quantities of toxin up to 1 mg. Possibly the enterotoxin released by the vibrios, on viable challenge, exceeded this amount, and overwhelmed the intestinal immunity. Due to the rapid binding of enterotoxin to mucosal cells, studies which quantitate the amount of enterotoxin released by V. cholerae in vivo are not possible. Secondly, as mentioned (p. 12) V. cholerae also produces substances such as mucinase, which allow penetration of the mucous layer in the intestine by the vibrios. Thus delivery of toxin to sensitive epithelial cells by vibrios in this location may be more efficient than simple injection into the lumen of the intestine. It may therefore be more difficult for intestinal antibodies to interrupt toxin-ganglioside binding.

The latter point raises the question as to whether it is valid to aim immunisation programmes at producing a purely antitoxic immunity. Holmgren and Svennerholm (1977) favoured a vaccination schedule which induced antitoxic and antibacterial immunity. They found a 100-fold higher degree of protection in rabbits against challenge with live vibrios resulted from immunisation with a combination of V. cholerae and enterotoxin, than resulted from immunisation with either of the two antigens alone. This effect was thought to be due to interference with two separate events in the pathogenesis of cholera, by antibacterial and antitoxic antibodies.

The serum antitoxin response obtained using vaccines containing V. cholerae organisms and toxoid (Table 16) was compared to that obtained after administration of toxoid alone (Table 14). With purely

oral immunisation the lack of serum antitoxin response was similar in both vaccine groups. However where i/m primary doses were administered the A.U./ml results were markedly lower with the combined vaccine group, despite the use of similar vaccination schedules and toxoid doses. The inclusion of V. cholerae in the vaccines might have been expected to enhance the antitoxin response due to the adjuvant action of the vibrio LPS (Verwey et al, 1975); however no evidence of this was obtained (Table 16).

There were a number of differences between the guinea-pigs used in Table 14 and Table 16, which may account for the results obtained. First, the oral immunisation of the latter group of animals was carried out without prior introduction of sodium bicarbonate into the drinking water for 18 hr. This was done to avoid the stimulation of acid secretion in the stomach which may result from prolonged exposure to bicarbonate. Although this may have affected the response to orally administered vaccine it could not possibly account for the observed decrease in serum antitoxin titre on i/m immunisation.

A second factor which may have influenced the response was the use of a different preparation of Arlacel A. It was not possible to obtain Arlacel from the original supplier and therefore a different product had to be purchased. The latter product is known to possess some different characteristics since it softened the gelatin capsules used for oral immunisation, which did not occur with the original Arlacel A preparation.

Thirdly, at this stage in the work the demand for guinea-pigs could not be met by the departmental stock, which supplied all other animals. Guinea-pigs were therefore purchased from Porcellus Animal Breeding Ltd., and for reasons of economy these were young animals of

200-250 g in weight. These animals did not tolerate transportation well, and took several weeks to adjust to their new environment and different diet. Thus, at the time of immunisation these guinea-pigs were considerably smaller than the group which received vaccines containing toxoid alone (Table 14), and were less well adapted to the animal house environment. These facts, perhaps with the influence of the other variables mentioned above, probably account for the poorer serum antitoxin response obtained in this group of guinea-pigs. It would seem obvious for future investigations to examine closely the effects of age and weight of animals and the nature of vaccine components.

Where V. cholerae organisms were included in the i/m primary vaccines (GP nos 491-499 and 533-541) precipitin lines against toxoid occurred with only a few sera (Table 18) and never where 3 months had elapsed between primary immunisation and withdrawal of blood. These results correlate with those obtained for A.U./ml (Tables 14 and 16) in which a distinct reduction in antitoxin titre was found in animals given an inoculum of V. cholerae organisms together with the toxoid.

Since it was not possible to agglutinate V. cholerae with the sera examined in this study, and since it was not possible to attribute protection against ileal loop challenge with V. cholerae to an anti-bacterial immune response, the results obtained for the complement fixation test were not surprising (Table 22). To reiterate the conclusion drawn above, the dose of V. cholerae used in this study was probably not sufficient to induce a significant antibacterial immune response. Thus antibacterial antibodies were not detected by protection tests or agglutination tests, and were barely detectable using the C.F.T. method.

The observation that V. cholerae alone gave a slightly improved C.F.T. response than V. cholerae + toxoid vaccines, is probably

explained by the reasons mentioned previously, since the latter vaccine was administered to those animals obtained from Porcellus Animal Breeding Ltd.

5. P.H.A. Test

The results obtained using this method of determining the anti-toxin content of sera, were rather variable when sera from animals of the same vaccine group were compared. The correlation between PHA titre and antiblueing activity of individual sera found by Finkelstein and Peterson (1970) was not evident in the present work. One of the dis-advantages of this method is that the amount of antigen fixed to the red cells at the coating stage varies on different occasions, even when the same antigen preparation at the same concentration is used. The degree of agglutination which occurs on addition of antibody is proportional to the amount of antigen coating the red cell. Thus the results obtained on replicate titrations of the same antisera tested against different coated-red-cell preparations may yield different titres. Apart from being extremely time saving, the major advantage of the Titertek apparatus is that it allows large numbers of sera to be tested against the same red-cell suspension, and therefore partly overcomes this problem.

A difficulty was also noted in the coating of red cells with different antigen preparations. The use of crude cholera toxin (N.I.H.) at a concentration of 1.0 mg/ml of sensitising solution was unsuccessful. This was possibly due to a concentration effect since a crude toxin preparation was used successfully by Hochstein, Feeley and Richardson (1970) to coat erythrocytes. These workers used a concentration of 200,000 B.D./ml of sensitising solution (which would require an equivalent amount of NIH toxin of 800 mg/ml) in comparison to the 250 B.D./ml

(1.0 mg/ml) used in this work. Since other antigens, e.g. ovalbumin are known to sensitise at the level of 200 μg/ml (Herbert, 1973) it was considered that 1000 μg/ml should be sufficient.

Cholera toxin purified by method C (see p. 107), at concentrations of 200 μg or 400 μg/ml of sensitising solution, was also used unsuccessfully in an attempt to coat erythrocytes for the P.H.A. test. Removal of contaminating antigens during purification should not interfere with haemagglutination by antisera raised against a purified toxoid preparation. The concentration of the toxin antigen in this preparation was also thought to account for the apparent failure of the antigen-coating process.

The $(NH_4)_2SO_4$-partially purified toxin preparation (see p. 88) yielded satisfactory results. Its use was supported by Hochstein et al (1970) who noted that the presence of somatic antigen in crude cholera toxin preparations did not interfere with the specificity of the PHA test.

It is stressed that owing to the indefinite nature and variability of cholera toxin-binding to erythrocytes, the results of the PHA test were considered a qualitative assessment of the serum antitoxin response to different vaccines. The results of the i/d blueing inhibition test were considered to give a more accurate indication of the antitoxin levels obtained.

6. General Discussion

Over the past few years there has been much research into producing a long-lasting immunity to cholera. Although a great variety of vaccines and vaccination schedules have been tested, no acceptable solution has emerged. The pathogenesis of the disease is now well documented and the development of disease symptoms has been averted by

many workers, either by preventing adhesion of vibrios to the mucosa, using whole cell or cell fraction vaccines, or by preventing enterotoxin-binding to epithelial cells, using toxoid vaccines. Unfortunately, the relative lack of knowledge, until recently, about gut-associated immune mechanisms, together with the difficulty in quantifying intestinal immunity has made the task of finding the most effective route and frequency of immunisation extremely difficult.

Fluid replacement therapy for cholera is now so effective that it is tempting to adopt this as the major line of defence against lethal infection, in preference to immunisation, particularly since fluid replacement is a more economical alternative than repeated immunisation of large sections of the community. However, the sheer volume of isotonic fluid required to treat each cholera patient (Carpenter, 1976) makes this a rather impractical therapy in epidemics. A more important point is that, although fluid replacement reduces mortality rates, it does nothing to decrease the cholera infection rate and maintains the source of infection in the community.

Cholera is one of the leading causes of morbidity in the developing countries at the present time, and as such a serious attempt to eradicate the disease should be made. In principle eradication should not be difficult; it requires the education of the public in personal hygiene. In practice, however, this is an immense task; a plentiful piped water supply to each household and an adequate sewerage system are two prerequisites of such an eradication programme. Obviously such developments, in rural India for example, are unlikely in the foreseeable future, and for this reason it seems worthwhile to pursue the search for an efficient vaccine, which, if incapable of eradicating the disease, will at least reduce the infection rate.

A number of recent developments in cholera research have introduced alternatives to the well tried, but relatively inefficient whole cell vaccine. A crucial step in the pathogenesis of cholera is the adhesion of vibrios to the mucosal surface of the gut. This may be prevented by the presence of antibacterial antibodies in the gut, which agglutinate the organisms in the lumen from where they are rapidly expelled (see p. 66). It has also come to light that motility of the vibrios is in some way involved in the colonisation of the intestine (see p. 13) and already workers such as Eubanks et al (1977) and Yang et al (1977) have suggested that vaccines containing flagellar fragments elicit protection against infection in experimental animals.

As mentioned previously, Pierce and co-workers have studied immunisation with toxoid preparations in depth, and have come to the conclusion that a parenteral-oral immunisation schedule is preferential, in terms of stimulating gut-associated immunity, to the purely parenteral cholera immunisation sequence used at present. However these workers noted a difficulty in the timing of administration of oral boosters (see p.184). As discussed this was possibly due to low levels of reversion of toxoid to active toxin and a subsequent effect on small lymphocytes. However, it is now possible to completely purify toxin B-subunits from the active subunit, thereby abolishing the risk of reversion to activity (Holmgren and Svennerholm, 1977).

The use of a combined vaccine containing V. cholerae LPS and enterotoxin was favoured by Svennerholm and Holmgren (1977) since administration of both antigens leads to greater protection than either antigen alone. However, the problem with using LPS as an immunogen, as is the case with whole cell vaccine, is the likelihood of inducing

immediate or delayed toxicity reactions at the site of injection, after repeated immunisation. Holmgren and Svennerholm (1977) suggested that it may be possible to isolate and perhaps synthesise the immuno-determinants of the LPS molecule which would be free of endotoxic activity. Thus combined vaccines containing enterotoxin B-subunits together with LPS-immunodeterminants or flagellar fragments, if appropriate, offer a hopeful solution to the cholera vaccine problem.

As suggested by Pierce et al (1977) the incorporation of vaccines in adjuvant mixtures may increase the duration of protection (a central problem in cholera immunisation) by stimulating higher peak antibody titres which take longer to fall below levels which afford protection. Thus if a reasonably innocuous adjuvant preparation can be found, this will be of great potential value.

As outlined by Stewart-Tull et al (1975) the injection of adjuvant mixtures containing heat-killed whole mycobacteria into the lungs of rabbits resulted in the formation of caseous lesions in the lung tissue, similar to the tuberculous cavities found in the lungs of tuberculous patients. No such lesions were produced by their water-soluble glycopeptide preparation, which also lacked the ability to induce polyarthritis in rats (Pearson and Wood, 1959; Watson, Pearson and Sharp, 1960) associated with other adjuvant-active substances, and the toxicity for mice associated with the presence of cord factor (Asselineau, Bloch and Lederer, 1953).

SUMMARY

The ultimate aim of this project was to produce immunity in guinea-pigs against ileal loop challenge with either V. cholerae enterotoxin or live organisms. Several parameters were examined in order to achieve this aim. First, the possibility of using the adjuvant-active, water-soluble glycopeptides, prepared from culture filtrates of Mycobacterium tuberculosis, was explored in immunisation schedules with cholera antigens. Second, the effect of the route of vaccine administration on the resultant immune response was studied, and third, the advantage of giving multiple doses of vaccine was considered.

Extensive studies on the i/p inoculation of mice with cholera toxoid demonstrated that the water-soluble glycopeptide, ST217, was indeed adjuvant-active, on injection with toxoid in a water-in-oil emulsion, and caused greatly increased serum antitoxin titres to be produced. The adjuvant effect of this water-soluble glycopeptide, on a weight basis, was superior to that obtained using whole mycobacterial cells, i.e. Freund's complete adjuvant.

Regarding the route of vaccine administration, it was noted that i/m immunisation of guinea-pigs caused the production of serum antibody and also levels of protection against ileal loop challenge with cholera enterotoxin. Oral immunisation did not allow the production of serum antibodies, however, slight protection against ileal challenge was evident, presumably due to the induction of an intestinal antibody response. The levels of protection noted after oral immunisation were, without exception, lower than those obtained on i/m administration of the same vaccine.

Where multiple immunisation schedules were employed the levels

of protection in guinea-pigs from ileal challenge with enterotoxin were improved by following an initial primary oral or i/m immunisation with an oral booster dose after 1 month. The administration of a second oral booster dose 2 months after the primary inoculation did not further boost the protective levels, which in all vaccine groups were lower than those obtained after the primary plus one booster dose.

The incorporation of the water-soluble glycopeptide, ST208, in the i/m primary inoculation mixture, resulted in increased levels of protection against enterotoxin challenge, as compared to toxoid in incomplete adjuvant. This increase in response was maintained throughout the three month period of observation.

Protection of ileal loops from challenge with viable V. cholerae was not evident in this study, regardless of vaccine content, or the route and frequency of administration.

REFERENCES

AGARWAL, S.C. & GANGULY, N.K. (1972). Oral immunisation with L-forms of Vibrio cholerae in human volunteers. Infect. Immun. 6, 17-20.

AHMED, A., BHATTACHARJEE, A.K. & MOSLEY, W. (1970). Characteristics of the serum vibriocidal and agglutinating antibodies in cholera cases and in normal residents of the endemic and non-endemic cholera areas. J.Immunol. 105, 431-441.

ALLWEISS, B., DOSTAL, J., CAREY, K.E., EDWARDS, T.F. & FRETER, R. (1977). The role of chemotaxis in the ecology of bacterial pathogens of mucosal surfaces. Nature 266, No. 5601 pp.448-450, Lond.

ALTIERI, P.L., BERMAN, S., GROFFINGER, A. & LOWENTHAL, J.P. (1970). Preparation of cholera vaccines by fluorocarbon extraction of cholera vibrio suspensions. Infect. Immun. 1, 334-337.

ASSELINEAU, J., BLOCH, H. & LEDERER, E. (1953). A toxic lipid component of the tubercle bacillus ("cord factor"). Am. Rev. Tuberc. 67, 853-858.

AZIZ, K.M.S., MOHSIN, A.K.M., HARE, W.K. & PHILLIPS, R.A. (1968). Using the rat as a cholera "model." Nature 220, No. 5169 pp.814-815, Lond.

AZIZ, K.M.S. & MOSLEY, W.H. (1972). Quantitative studies of toxin in the stools and jejunal aspirates of patients with cholera. J. infect. Dis. 125, 36-44.

AZURIN, J.C., CRUZ, A., PESIGAN, T.P., ALVERO, M., CAMENA, T., SUPLIDO, R., LEDESMA, L. & GOMEZ, C.Z. (1967). A controlled field trial of the effectiveness of cholera and cholera El Tor vaccines in the Philippines. Bull. Wld. Hlth. Org. 37, 703-727.

BANWELL, J.G., PIERCE, N.F., MITRA, R.C., BRIGHAM, K.L., CARANASOS, G.J., KEIMOWITZ, R.I., FEDSON, D.S., THOMAS, J., GORBACH, S.L., SACK, R.B. & MONDAL, A. (1970). Intestinal fluid and electrolyte transport in human cholera. J. clin. Invest. 49, 183-195.

BART, K.J., HUQ, Z., KHAN, M. & MOSLEY, W.H. (1970). Sero-epidemiologic studies during a simultaneous epidemic of infection with El Tor Ogawa and Classical Inaba Vibrio cholerae. J. infect. Dis. 121, S17-S24.

BARUA, D. & SACK, R.B. (1964). Serological studies on cholera. Indian J. med. Res. 52, 855-866.

BASU, S. & PICKET, M.J. (1969). Reaction of Vibrio cholerae and choleragenic toxin in ileal loops of laboratory animals. J. Bact. 100, 1142-1143.

BEH, K.J. (1977). The origin of IgA-containing cells in intestinal lymph of sheep. Aust. J. exp. Biol. med. Sci. 55, 263-274.

BENENSON, A.S., JOSEPH, R.P. & OSEASOHN, R.O. (1968,a). Cholera vaccine field trials in East Pakistan. 1. Reaction and antigenicity studies. Bull. Wld Hlth Org. 38, 347-357.

BENENSON, A.S., MOSLEY, W.H., FAHIMUDDIN, M. & OSEASOHN, R.O. (1968,b). Cholera vaccine field trials in East Pakistan. 2. Effectiveness in the field. Bull. Wld Hlth Org. 38, 359-372.

BENENSON, A.S., SAAD, A. & PAUL, M. (1968,c). Serological studies in cholera. 1. Vibrio agglutinin response of cholera patients determined by a microtechnique. Bull. Wld Hlth Org. 38, 267-276.

BENENSON, A.S., SAAD, A. & MOSLEY, W.H. (1968,d). Serological studies in cholera. 2. The vibriocidal antibody response of cholera patients determined by a microtechnique. Bull. Wld Hlth Org. 38, 277-285.

BENENSON, A.S., SAAD, A., MOSLEY, W.H. & AHMED, A. (1968,e). Serological studies in cholera. 3. Serum toxin neutralisation - rise in titre in response to infection with V. cholerae and the level in the "normal" population in East Pakistan. Bull. Wld Hlth Org. 38, 287-295.

BENNETT, B. & CUATRECASAS, P. (1975). Mechanism of activation of adenylate cyclase by Vibrio cholerae enterotoxin. J. memb. Biol. 22, 29-52.

BENNET, V. O'KEEFE, E. & CUATRECASAS, P. (1975). Mechanism of action of cholera toxin and the mobile receptor theory of hormone receptor-adenylate cyclase interactions. Proc. natn. Acad. Sci. U.S.A. 72, 33-37.

BENNET, V., CRAIG, S., HOLLENBERG, M.D., O'KEEFE, E., SAHYOUN, N. & CUATRECASAS, P. (1976). Structure and function of cholera toxin and hormone receptors. J. Supramolec. Struct. 4, 99-120.

BERGEY (1974). Bergey's Manual of Determinative Microbiology, Eighth Edition. pp.340-345. ed. Buchanan, R.E. & Gibbons, N.E. The Williams & Wilkins Company, Baltimore.

BERKENBILE, F. & DELANEY, R. (1976). Stimulation of adenylate cyclase by Vibrio cholerae toxin and its active subunit. J. infect. Dis. 133, S82-S88.

BERRY, L.J. (1975). Bacterial toxins : Cholera enterotoxin. Lloydia, 38, 8-20.

BHATTACHARYA, P. & MUKERJEE, S. (1968). Production of antibodies after live enteral cholera vaccination. J. infect. Dis. 118, 271-277.

BHATTACHARYA, P., NARAYANASWAN, A. & MUKERJEE, S. (1968). Production of antitoxic immunity by live oral cholera vaccine. J. Bact. 95, 255-256.

BURROWS, W. (1968). Cholera toxins. A. Rev. Microbiol. 22, 245-268.

BURROWS, W., ELLIOT, M.E. & HAVENS, I. (1947). Studies on immunity to Asiatic cholera. IV. The excretion of copro antibody in experimental enteric cholera in the guinea-pig. J. infect. Dis. 81, 261-281.

BURROWS, W. & MUSTEIKIS, G.M. (1966). Cholera infection and toxin in the rabbit ileal loop. J. infect. Dis. 116, 183-190.

BURROWS, W., KAUR, J. & CERCAVISKI, L. (1971). Discussion: The cholera enterotoxin and local immunity. Ann. N.Y. Acad. Sci. 176, 323-329.

BURROWS, W. & KAUR, J. (1975). The antibody basis of local immunity to experimental cholera infection in the rabbit ileal loop. Prog. Drug Res. 19, 471-480.

CALLAHAN, L.T., RYDER, R.C. & RICHARDSON, S.H. (1971). Biochemistry of Vibrio cholerae virulence. II Skin permeability factor/cholera enterotoxin production in a chemically defined medium. Infect. Immun. 4, 611-618.

CARPENTER, C.C.J. (1970). Pathogenesis and pathophysiology of cholera. In Principles & Practice of Cholera Control. Public Health Publication No. 40. pp.53-56. World Health Organisation, Geneva.

CARPENTER, C.C.J., SACK, R.B., FEELEY, J.C. & STEENBERG, R.W. (1968). Site and characteristics of electrolyte loss and effect of intraluminal glucose in experimental canine cholera. J. clin. Invest. 47, 1210-1220.

CARPENTER, C.C.J., GREENOUGH, W.B. & GORDON, R.S. (1974). Pathogenesis and pathophysiology of cholera. In Cholera, chapter 6, ed. Barua, D. & Burrows, W., W.B. Saunders Company.

CARPENTER, C.C.J. (1976). Treatment of cholera-tradition and authority versus science, reason and humanity. The John Hopkins Med. J. 139, 153-162.

CASH, R.A., MUSIC, S.I., LIBONATI, J.P., SCHWARTZ, A.R. & HORNICK, R.B. (1974). Live oral cholera vaccine: Evaluation of the clinical effectiveness of two strains in humans. Infect. Immun. 10, 762-764.

CEDGÅRD, S., HALLBÄCK, D-A., JODAL, M., LUNDGREN, O. & REDFORS, S. (1978). The effects of cholera toxin on intramural blood flow distribution and capillary hydraulic conductivity in the cat small intestine. Acta physiol. scand. 102, 148-158.

CHISARI, F.V. & NORTHRUP, R.S. (1974). Pathophysiologic effects of lethal and immunoregulatory doses of cholera enterotoxin in the mouse. J. Immunol. 113, 740-749.

CHISARI, F.V., NORTHRUP, R.S. & CHEN, L.C. (1974). The modulating effect of cholera enterotoxin on the immune response. J. Immunol. 113, 729-739.

COLEMAN, W.H., KAUR, J., IWERT, M.E., KASAI, G.J. & BURROWS, W. (1968). Cholera toxins: Purification and preliminary characterisation of ileal loop reactive type 2 toxin. J. Bact. 96, 1137-1143.

CRAIG, J.P. (1965). A permeability factor (toxin) found in cholera stools and culture filtrates and its neutralisation by convalescent cholera sera. Nature 207, No. 4997, pp.14-16, Lond.

CRAIG, J.P. (1966). Preparation of the vascular permeability factor of Vibrio cholerae. J. Bact. 92, 793-795.

CRAIG, J.P. (1968). Unpublished experiments. See Microbial Toxins vol. IIA, ed. Kadis, S., Montie, T.C. & Ajl, S.J. (1971). Academic Press, New York and London.

CRAIG, J.P. (1970). Some observations on the neutralisation of cholera vascular permeability factor in vivo. J. infect. Dis. 121, S100-S109.

CRAIG, S.W. & CEBRA, J.J. (1973). Immunoglobulin A formation in the gastrointestinal tract and peripheralization of the immunocytes and immunoglobulin to other sites. In Proceedings of the symposium on new approaches for inducing natural immunity to pyogenic organisms. D.H.E.W. Publication no. (N.I.H.) 74-553 pp.85-90.

CRAIG, S.W. & CUATRECASAS, P. (1975). Mobility of cholera toxin receptors on rat lymphocyte membranes. Proc. natn. Acad. Sci. U.S.A. 72, 3844-3848.

CRAIG, S.W. & CUATRECASAS, P. (1976). Immunological probes into the mechanism of cholera toxin action. Immunol. Communs. 5, 387-400.

CUATRECASAS, P. (1973,a). The interaction of Vibrio cholerae toxin with cell membranes. Biochemistry 12, 3547-3557.

CUATRECASAS, P. (1973,b). Gangliosides and membrane receptors for cholera toxin. Biochemistry 12, 3558-3566.

CUATRECASAS, P. (1973,c). Cholera toxin-fat cell interaction and the mechanism of activation of the lipolytic response. Biochemistry 12, 3567-3577.

CUATRECASAS, P. (1973,d). Vibrio cholerae choleragenoid. Mechanism of inhibition of cholera toxin action. Biochemistry 12, 3577-3581.

CUATRECASAS, P., PARIKH, I. & HOLLENBERG, M.D. (1973). Affinity chromatography & structural analysis of Vibrio cholerae enterotoxin-ganglioside agarose and the biological effects of ganglioside-containing soluble polymers. Biochemistry 12, 4253-4264.

CURLIN, G.T., MOSLEY, W.H. & GREENOUGH, W.B. (1973). Cholera antitoxin titrations: A comparative study of fat cell, ileal loop and rabbit skin assays. J. infect. Dis. 127, 294-298.

CVJETANOVIC, B. (1975). Contribution of Haffkine to the concept and practice of controlled field trials of vaccines. Prog. Drug Res. 19, 481-489.

DAS GUPTA, A., SINHA, R., SHRIVASTAVA, D.L., DE, S.P., TANEJA, B.L., RAO, M.S. & ABOU-GAREEB, A.H. (1967). Controlled field trial of the effectiveness of cholera and cholera El Tor vaccines in Calcutta. Bull. Wld Hlth Org. 37, 371-385.

DE, S.N. (1959). Enterotoxicity of bacteria-free culture filtrates of Vibrio cholerae. Nature 183, 1533-1534, Lond.

DE, S.N. & CHATTERJE, D.N. (1953). An experimental study of the mechanism of action of Vibrio cholerae on the intestinal mucous membrane. J. Path. Bact. 66, 559-562.

DE, S.N., GHOSE, M.L. & SEN, A. (1960). Activities of bacteria-free preparations from Vibrio cholerae. J. Path. Bact. 79, 373-380.

DE JONGE, H.R. (1975). The response of small intestinal villous and crypt epithelium to cholera toxin in rat and guinea-pig. Evidence against a specific role of the crypt cells in choleragen-induced secretion. Biochim. biophys. Acta. 381, 128-143.

DOUGLAS, S.D., ZUCKERMAN, S.H. & OOKA, M.P. (1976). Effects of cholera exotoxin on the mitochondrial morphology of lymphoid cells. Lab. Invest. 35, 607-614.

DUGUID, J.P. & GILLIES, R.R. (1957). Fimbriae and adhesive properties in dysentery bacilli. J. Path. Bact. 74, 397-411.

DUHAMEL, R.C., TALBOT, P. & GRADY, G.F. (1970). Production, purification and assay of cholera enterotoxin. J. infect. Dis. 121, S85-S91.

DURIKHIN, K.V., POPOVA, A.E. & KOBRINSKII, J.D. (1976). Choleragen and neuraminidase production by cholera vibrios in vitro. Bull. exp. Biol. & Med. 81, 707-709.

DUTTA, N.K., PANSE, M.V. & KULKARNI, D.R. (1959). Role of cholera toxin in experimental cholera. J. Bact. 78, 594-595.

ELLIOT, H.L., CARPENTER, C.C.J., SACK, R.B. & YARDLEY, J.H. (1970). Small bowel morphology in experimental canine cholera. A light microscopic study. Lab. Invest. 22, 112-120.

EUBANKS, E.R., GUENTZEL, M.N. & BERRY, L.J. (1977). Evaluation of surface components of Vibrio cholerae as protective immunogens. Infect. Immun. 15, 533-538.

EVANS, D.J. & RICHARDSON, S.H. (1968). In vitro production of choleragen and vascular permeability factor by Vibrio cholerae. J. Bact. 96, 126-130.

FEELEY, J.C. & ROBERTS, C.O. (1969). Immunological responses of laboratory animals to cholera vaccines, toxin and toxoid. Tex. Rep. Biol. Med. 27, suppl. 1, 181-201.

FELGNER, P., BÜCKEN, W. & RICHTER, H.M. (1975). Further studies on the behaviour of 2-mercaptoethanol resistant antibody following cholera vaccination in a non-endemic country. Zbl. Bakt. Hyg. 233, 531-535.

FIELD, M. (1976). Regulation of active ion transport in the small intestine. Ciba Foundation Symp. 42, 109-128.

FINKELSTEIN, R.A. (1965). Immunological aspects of experimental cholera. In Proc. Cholera Res. Symp. Pub. Hlth. Serv. Publication No. 1328. pp. 58-63. U.S. Government Printing Office, Washington, D.C.

FINKELSTEIN, R.A. (1969). The role of choleragen in the pathogenesis and immunology of cholera. Tex. Rep. Biol. Med. 27, suppl. 1, 181-201.

FINKELSTEIN, R.A. (1970). Antitoxic immunity in experimental cholera: Observations with purified antigens and the ligated ileal loop model. Infect. Immun. 1, 464-467.

FINKELSTEIN, R.A. (1972). Properties of cholera exo-enterotoxin (choleragen) and its natural toxoid (choleragenoid). Toxicon, 10, 441-450.

FINKELSTEIN, R.A. (1973). Cholera. Crit. Revs. Microbiol. 2, 553-623.

FINKELSTEIN, R.A. (1976). Possibility of immunisation against cholera and related enteropathies. Dev. Biol. Stand. 33, 102-107.

FINKELSTEIN, R.A. & MUKERJEE, S. (1963). Haemagglutination, a rapid method for differentiating Vibrio cholerae and El Tor vibrios. Proc. Soc. exp. Biol. Med. 112, 355-359.

FINKELSTEIN, R.A., ATTHASAMPUNNA, P., CHULASAMAYA, M. & CHARUNMETHEE, P. (1966). Pathogenesis of experimental cholera: Biologic activities of purified procholeragen A. J. Immunol. 96, 440-449.

FINKELSTEIN, R.A. & ATTHASAMPUNNA, P. (1967). Immunity against experimental cholera. Proc. Soc. exp. Biol. Med. 125, 465-469.

FINKELSTEIN, R.A., JEHL, J.J. & GOTH, A. (1969). Pathogenesis of experimental cholera: Choleragen-induced rat foot edema; a method of screening anticholera drugs. Proc. Soc. exp. Biol. Med. 132, 835-840.

FINKELSTEIN, R.A. & LoSPALLUTO, J.J. (1969). Pathogenesis of experimental cholera. Preparation and isolation of choleragen and choleragenoid. J. exp. Med. 130, 185-202.

FINKELSTEIN, R.A. & HOLLINGSWORTH, R.C. (1970). Antitoxic immunity in experimental cholera: Observations with purified antigens and the rat foot edema model. Infect. Immun. 1, 468-473.

FINKELSTEIN, R.A. & LoSPALLUTO, J.J. (1970). Production of highly purified choleragen and choleragenoid. J. infect. Dis. 121, S63-S72.

FINKELSTEIN, R.A. & PETERSON, J.W. (1970). In vitro detection of antibody to cholera enterotoxin in cholera patients and laboratory animals. Infect. Immun. 1, 21-29.

FINKELSTEIN, R.A., PETERSON, J.W. & LoSPALLUTO, J.J. (1971,a). Conversion of cholera exo-enterotoxin (choleragen) to natural toxoid (choleragenoid). J. Immunol. 106, 868-871.

FINKELSTEIN, R.A., FUJITA, K. & LoSPALLUTO, J.J. (1971,b). Procholeragenoid: an aggregated intermediate in the formation of choleragenoid. J. Immunol. 107, 1043-1051.

FINKELSTEIN, R.A., LaRUE, M.K. & LoSPALLUTO, J.J. (1972). Properties of the cholera exo-enterotoxin: effects of dispersing agents and reducing agents in gel filtration and electrophoresis. Infect. Immun. 6, 934-944.

FINKELSTEIN, R.A., BOESMAN, M., NEOH, S.H., LaRUE, M.K. & DELANEY, R. (1974). Dissociation and recombination of the subunits of the cholera enterotoxin (choleragen). J. Immunol. 113, 145-150.

FLORES, J. & SHARP, G.W.G. (1976). The activation of adenylate cyclase by cholera toxin: Possible interaction with the nucleotide regulatory site. Ciba Foundation Symp. 42, 89-108.

FORMAL, S.B., KUNDEL, D., SCHNEIDER, H., KUNEV, N. & SPRINZ, H. (1961). Studies with Vibrio cholerae in the ligated loop of the rabbit intestine. Br. J. exp. Path. 42, 504-510.

FRETER, R. (1969). Studies on the mechanism of action of intestinal antibody in experimental cholera. Tex. Rep. Biol. Med. 27, suppl. 1, 299-316.

FRETER, R. (1970). Mechanism of action of intestinal antibody in experimental cholera. II Antibody-mediated antibacterial reaction at the mucosal surface. Infect. Immun. 2, 556-562.

FRETER, R. (1972). Parameters affecting the association of vibrios with the intestinal surface in experimental cholera. Infect. Immun. 6, 134-141.

FRETER, R. & JONES, G.W. (1976). Adhesive properties of Vibrio cholerae: Nature of the interaction with intact mucosal surfaces. Infect. Immun. 14, 246-256.

FROST, W.H. (1976). Cholera: Synopsis of clinical aspects and principles of treatment. Can. med. Ass. J. 115, 401-403.

FUHRMAN, G.J. & FUHRMAN, F.A. (1960). Inhibition of active sodium transport by cholera toxin. Nature, 188, 71-72, Lond.

FUHRMAN, F.A., FUHRMAN, G.J. & BURROWS, W. (1962). Action and properties of an inhibitor of active transport of sodium produced by cholera vibrios. J. infect. Dis. 111, 225-232.

FUJITA, K. & FINKELSTEIN, R.A. (1972). Antitoxic immunity in experimental cholera: Comparison of immunity induced perorally and parenterally in mice. J. infect. Dis. 125, 647-655.

GALLUT, J. (1962). Studies on the vibrio antigen "O" complex. II Spontaneous selective liberation of V. cholerae and V. eltor cell walls, with loss of O agglutinability. Extraction of the specific agglutinogen. Annales de l'Institute Pasteur, 102, 309-327.

GALLUT, J. (1965). Antigenic structure of gibrios. In Proc. Cholera Res. Symp., Pub. Hlth. Serv. Publication No. 1328, pp.235-243. U.S. Government Printing Office, Washington, D.C.

GAN, K.H. & TJIA, S.K. (1963). A new method for the differentiation of Vibrio comma and Vibrio eltor. Am. J. Hyg. 77, 184-186.

GANGAROSA, E.J., BEISEL, W.R., BENYAJATA, C., SPRINZ, H. & PIYARATN, P. (1960). The nature of the gastrointestinal lesion in Asiatic cholerae and its relation to pathogenesis. A biopsy study. Am. J. trop. Med. 9, 125-135.

GARDNER, A.D. & VENKATRAMAN, K.V. (1935). The antigens of the cholera group of vibrios. J. Hyg. 35, 262-282.

GERMANIER, R. FÜRER, E., VARALLYAY, S. & INDERBITZIN, T.M. (1977). Antigenicity of cholera toxoid in humans. J. infect. Dis. 135, 512-516.

GILL, D.M. (1976). The arrangement of subunits in cholera toxin. Biochemistry, 15, 1242-1248.

GRADY, G.F., MADOFF, M.A., DUHAMEL, R.C., MOORE, E.W. & CHALMERS, T.C. (1968). Sodium transport inhibition by cholera toxin: The role of non-ionic diffusion of ammonia. J. infect. Dis. 118, 263-270.

GRADY, G.F. & CHANG, M.C. (1970). Cholera enterotoxin free from permeability factor? J. infect. Dis. 121, S92-S95.

GRADY, G.F., KEUSCH, G.T. & DESCHNER, E.E. (1975). Kinetics of absorption of toxin of Vibrio cholerae. J. infect. Dis. 131, 210-216.

GREENOUGH, W.B., PIERCE, N.F. & VAUGHAN, M. (1970). Titration of cholera enterotoxin and antitoxin in isolated fat cells. J. infect. Dis. 121, S111-S113.

GUENTZEL, M.N. & BERRY, L.J. (1975). Motility as a virulence factor for Vibrio cholerae. Infect. Immun. 11, 890-897.

GUY-GRAND, D., GRISCELLI, C. & VASSALLI, P. (1975). Peyer's patches, gut IgA plasma cells and thymic function: Study in nude mice bearing thymic grafts. J. Immunol. 115, 361-364.

HALL, J.G., HOPKINS, J. & ORLANS, E. (1977). Migration pathways of lymphoid cells with reference to the gut immunoglobulins and lymphomata. Biochem. Soc. Trans. 5, 1581-1583.

HECKLY, R.J. & WOLOCHOW, H. (1970). Characterisation and purification of cholera toxin. J. infect. Dis. 121, S80-S84.

HERBERT, W.J. (1973). Passive haemagglutination. In Handbook of Experimental Immunology, Chapter 21. Ed. Weir, D.M. Blackwell Scientific Publications, Oxford and Edinburgh.

HIRSCHHORN, N., PIERCE, N.F., KOBARI, K. & CARPENTER, C.C.J. (1974). The treatment of cholera. Cholera, chapter 12, ed. Barua, D. and Burrows, W., W.B. Saunders Company.

HOCHSTEIN, H.D., FEELEY, J.C. & RICHARDSON, S.H. (1970). Titration of cholera antitoxin levels by passive haemagglutination tests using fresh and formalinised sheep erythrocytes. Proc. Soc. exp. Biol. 133, 120-124.

HOLLENBERG, M.D., FISHMAN, P.H., BENNET, V. & CUATRECASAS, P. (1974). Cholera toxin and cell growth: Growth of membrane gangliosides. Proc. natn. Acad. Sci. U.S.A. 71, 4224-4228.

HOLMGREN, J., LÖNNROTH, I. & OUCHTERLONY, Ö. (1971). Immunochemical studies of two cholera toxin-standard culture filtrate preparations of Vibrio cholerae. Infect. Immun. 3, 747-755.

HOLMGREN, J., ANDERSSON, Å., WALLERSTROM, G. & OUCHTERLONY, Ö. (1972). Experimental studies on cholera immunisation. II. Evidence for protective antitoxic immunity mediated by serum antibodies as well as local antibodies. Infect. Immun. 6, 662-667.

HOLMGREN, J., LINDHOLM, L. & LÖNNROTH, I. (1974). Interaction of cholera toxin and toxin derivatives with lymphocytes. 1. Binding properties and interference with lectin-induced cellular stimulation. J. exp. Med. 139, 801-819.

HOLMGREN, J. & LÖNNROTH, I. (1975). Oligomeric structure of cholera toxin: characterisation of the H & L subunits. J. Gen. Micro. 86, 49-65.

HOLMGREN, J., LÖNNROTH, I., MÄNSSON, J.E. & SVENNERHOLM, L. (1975,a). Interaction of cholera toxin and membrane Gm_1 ganglioside of small intestine. Proc. natn. Acad. Sci. U.S.A. 72, 2520-2524.

HOLMGREN, J., SVENNERHOLM, A.M., OUCHTERLONY, Ö., ANDERSSON, Å., WALLERSTRÖM, G. & WESTERBERG-BERNDTSSON, U. (1975,b). Antitoxic immunity in experimental cholera: protection, and serum and local antibody responses in rabbits after enteral and parenteral immunisation. Infect. Immun. 12, 1331-1340.

HOLMGREN, J. & LINDHOLM, L. (1976). Cholera toxin, ganglioside receptors and the immune response. Immunol. Communs. 5, 737-758.

HOLMGREN, J. & LÖNNROTH, I. (1976). Cholera toxin and the adenylate cyclase-activating signal. J. infect. Dis. 133, S64-S74.

HOLMGREN, J. & SVENNERHOLM, A. (1977). Mechanisms of disease and immunity in cholera: a review. J. infect. Dis. 136, S105-S112.

HOLMGREN, J., SVENNERHOLM, A.-M., LÖNNROTH, I., FALL-PERSSON, M., MARKMAN, B. & LUNDBECK, H. (1977). Development of improved cholera vaccine based on subunit toxoid. Nature, 269, No. 5629, 602-604, Lond.

HORNICK, R.B., MUSIC, S.I., WENZEL, R., CASH, R., LIBONATI, J.P., SNYDER, M.J. & WOODWARD, T.E. (1971). The Broad Street pump revisited: Response of volunteers to injested cholera vibrios. Bull. N.Y. Acad. Med. 47, 1169-1180.

HORNICK, R.B., MUSIC, S.I., LIBONATI, J.P., SNYDER, M.J. & WOODWARD, T.E. (1972). Cholera in volunteers, In Symposium on Cholera, Woods Hole, Mass., 1971. Proc. 7th Joint Conference U.S.-Japan Cooperative Med. Sci. Prog. Cholera Panel. Nat. Inst. Hlth., Bethesda, M.D. pp.37-38.

HUBER, G.S. & PHILLIPS, R.A. (1960). Cholera and the sodium pump. Research Report MR 005.09-1040.1.7. Bureau of Med. & Surgery. Dept. of the Navy, Washington, D.C.

IWERT, M.E., LEITCH, G.J. & BURROWS, W. (1967). Increased serum glutamic-oxalacetic transaminase and lactic dehydrogenase levels produced by cholera infection and cell-free toxin in the rabbit. J. infect. Dis. 117, 265-272.

IWERT, M.E., DEB, B.C., SHRIVASTAVA, D.L. & BURROWS, W. (1968). Cholera toxins: Increased levels of serum glutamic-oxalacetic transaminase in human cholera. J. infect. Dis. 118, 422-426.

JACKSON, G.D.F. & REDMOND, J.W. (1971). Immunochemical studies of the O-antigens of Vibrio cholerae 569B (Inaba). F.E.B.S. Letters, 13, 117-120.

JACOBS, J.W., NIALL, H.D. & SHARP, G.W.G. (1974). The amino terminal sequence of cholera toxin subunits. Biochem. biophys. Res. Commun. 61, 391-395.

JENKIN, C.R. & ROWLEY, D. (1960). The importance of antibody in the prevention of experimental cholera in rabbits. Br. J. exp. Pathol. 41, 24-30.

JENSEN, R., GREGORY, B., NAYLOR, J. & ACTOR, P. (1972). Isolation of protective somatic antigen from Vibrio cholerae (Ogawa) ribosomal preparations. Infect. Immun. 6, 156-161.

JOHNSON, G.L. & BOURNE, H.R. (1977). Influence of cholera toxin on the regulation of adenylate cyclase by GTP. Biochem. biophys. Res. Commun. 78, 792-798.

JONES, G.W., ABRAMS, G.D. & FRETER, R. (1976). Adhesive properties of Vibrio cholerae: Adhesion to isolated brush border membranes and haemagglutinating activity. Infect. Immun. 14, 232-239.

JONES, G.W. & FRETER, R. (1976). Adhesive properties of Vibrio cholerae: Nature of the interaction with isolated rabbit brush border membranes. Infect. Immun. 14, 240-245.

JOÓ, I., CSIZÉR, Z. & JUHÁSZ, U.P. (1972). Immune response to plain and adsorbed cholera vaccines. Prog. immunobiol. Stand. 5, 350-354.

KANFER, J.N., CARTER, T.P. & KATZEN, H.M. (1976). Lipolytic action of cholera toxin on fat cells. Re-examination of the concept implicating Gm_1 ganglioside as the native membrane receptor. J. biol. Chem. 251, 7610-7619.

KASAI, G.J. & BURROWS, W. (1966). The titration of cholera toxin and antitoxin in the rabbit ileal loop. J. infect. Dis. 116, 606-614.

KATELEY, J.R. & FRIEDMAN, H. (1975). Modulation of antibody synthesis by cholera exotoxin: Influence on helper thymocytes. Cell. Immunol. 18, 239-245.

KAUFFMANN, F. (1950). On the serology of the Vibrio cholerae. Acta path. microbiol. scand. 27, 283-299.

KAUR, J. & SHRIVASTAV, J.B. (1964). Immunochemical studies on vibrio polysaccharides. Indian J. med. Res. 52, 809-816.

KAUR, J., BURROWS, W. & CERCAVISKI, L. (1969). Cholera toxins: immunogenicity of the rabbit ileal loop toxin and related antigens. J. Bact. 100, 985-993.

KAUR, J., KÖNIG, H.C., MARTIN, W.R. & BURROWS, W. (1970). The extractable lipid of the cholera enterotoxin. J. infect. Dis. 121, 78-80.

KAUR, J., BURROWS,W. & FURLONG, M.A. (1971). Immunity to cholera: antibody response in the lower ileum of the rabbit. J. infect. Dis. 124, 359-366.

KAUR, J., McGHEE, R. & BURROWS, W. (1972). Immunity to cholera: the occurrence and nature of the antibody-active immunoglobulins in the lower ileum of the rabbit. J. Immunol. 108, 387-395.

KIMBERG, D.V., FIELD, M., JOHNSON, J., HENDERSON, A. & GERSHON, E. (1971). Stimulation of intestinal mucosal adenyl cyclase by cholera enterotoxin and prostaglandins. J. clin. Invest. 50, 1218-1230.

KING, A.A. (1957). The preparation of plates for the gel diffusion precipitin test. J. med. Lab. Techn. 14, 234-238.

KLAPPER, D.G., FINKELSTEIN, R.A. & CAPRA, J.D. (1976). Subunit structure and N-terminal amino acid sequence of the three chains of cholera enterotoxin. Immunochemistry, 13, 605-611.

KUROSKY, A., MARKEL, D.E., TOUCHSTONE, B. & PETERSON, J.W. (1976). Chemical characterisation of the structure of cholera toxin and its natural toxoid. J. infect. Dis. 133, S14-S22.

KUSAMA, H. & CRAIG, J.P. (1970). Production of biologically active substances by two strains of Vibrio cholerae. Infect. Immun. 1, 80-87.

LAI, C.Y. (1977). Determination of the primary structure of cholera toxin B-subunit. J. biol. Chem. 252, 7249-7256.

LAI, C.Y., MENDEZ, E. & CHANG, D. (1976). Chemistry of cholera toxin: the subunit structure. J. infect. Dis. 133, S23-S30.

LAI, C.Y., MENDEZ, E., CHANG, D. & WANG, M. (1977). Primary structure of cholera toxin B-subunit. Biochem. biophys. Res. Comm. 74, 215-222.

LANKFORD, C.E. (1960). Factors of virulence of Vibrio cholerae. Ann. N.Y. Acad. Sci. 88, 1203-1212.

LEDLEY, F.D., MULLIN, B.R., LEE, G., ALOJ, S.M., FISHMAN, P.H., HUNT, L.T., DAYHOFF, M.O. & KOHN, L.D. (1976). Sequence similarity between cholera toxin and glycoprotein hormones: Implications for structure activity relationship and mechanism of action. Biochem. biophys. Res. Comm. 69, 852-859.

LEITCH, G.J. & BURROWS, W. (1968). Experimental cholera in the rabbit ligated intestine: ion and water accumulation in the duodenum, ileum and colon. J. infect. Dis. 118, 349-359.

LEVINSON, S.L. & BLUME, A.J. (1977). Altered guanine nucleotide hydrolysis as basis for increased adenylate cyclase activity after cholera toxin treatment. J. biol. Chem. 252, 3766-3774.

LEWIS, A.C. & FREEMAN, B.A. (1969). Separation of type 2 toxins of Vibrio cholerae. Science 165, 808-809.

LEWIS, A.C., RICHARDSON, S.H. & SHERIDAN, B. (1976). Biochemistry of Vibrio cholerae virulence: Purification of cholera enterotoxin by preparative disc electrophoresis. Appl. environ. Micro. 32, 288-293.

LINDHOLM, L., HOLMGREN, J., LANGE, S. & LÖNNROTH, I. (1977). Interaction of cholera toxin and toxin derivatives with lymphocytes. II. Modulating effects of cholera toxin on in vivo humoral and cellular immune responses. Int. Archs. Allergy appl. Immun. 50, 555-573.

LÖNNROTH, I. & HOLMGREN, J. (1973). Subunit structure of cholera toxin. J. gen. Micro. 76, 417-427.

LÖNNROTH, I. & HOLMGREN, J. (1974). Chemical modification of cholera toxin for characterisation of antigenic, receptor-binding and toxic sites. F.E.B.S. Letters, 44, 282-285.

LÖNNROTH, I. & HOLMGREN, J. (1975). Protein reagent modification of cholera toxin: characterisation of effects on antigenic, receptor-binding and toxic properties. J. gen. Micro. 91, 263-277.

LÖNNROTH, I. & LÖNNROTH, C. (1977). Interaction of cholera toxin and its subunits with lymphocytes. Expl. Cell. Res. 104, 15-24.

LoSPALLUTO, J.J. & FINKELSTEIN, R.A. (1972). Chemical and physical properties of cholera exo-enterotoxin (choleragen) and its spontaneously formed toxoid (choleragenoid). Biochim. biophys. Acta. 257, 158-166.

LYLES, S.T. & GARDNER, E.W. (1958). Studies on the antigenic structure of Vibrio cholerae. I. The effects of heat on the agglutinating antigens. J. infect. Dis. 103, 67-74.

MAHALANABIS, D., WATTEN, R.H. & WALLACE, C.K. (1974). Clinical aspects and management of pediatric cholera. In Cholera, chapter 11, ed. Barua, D. & Burrows, W., W.B. Saunders Company.

MARSH, M.N. (1975). Studies of intestinal lymphoid tissue. I. Electron microscopic evidence of "blast transformation" in epithelial lymphocytes of mouse small intestinal mucosa. Gut 16, 665-682.

MARTIN, A.R., VERNON, T.J. & MOSLEY, W.H. (1969). Neutralisation of the vascular permeability factor of Vibrio cholerae in man. Am. J. trop. Med. 18, 253-257.

MARTIN, B.R., HOUSLEY, M.D. & KENNEDY, E.L. (1977). Cholera toxin requires oxidised nicotinamide adenine dinucleotide to activate adenylate cyclase in purified rat liver plasma membranes. Biochem. J. 161, 639-642.

McCORMACK, W.M., RAHMAN, A.S.M.M., CHOWDHURY, A.K.M.A., MOSLEY, W.H. & PHILLIPS, R.A. (1969). Report on the 1966-67 cholera vaccine field trial in rural East Pakistan. 3. The lack of effect of prior vaccination or circulating vibriocidal antibody on the severity of clinical cholera. Bull. Wld Hlth Org. 40, 199-204.

McWILLIAMS, M., PHILLIPS-QUAGLIATA, J.M. & LAMM, M.E. (1977). Mesenteric lymph node B-lymphoblasts, which home to the small intestine are pre-committed to IgA synthesis. J. exp. Med. 145, 866-875.

MENDEZ, E., LAI, C.Y. & WODNAR-FILIPOWICZ, A. (1975). Location and the primary structure around the disulphide bonds in cholera toxin. Biochem. biophys. Res. Comm. 67, 1435-1443.

MERRIT, C.B. & SACK, R.B. (1970). Sensitivity of agglutinating and vibriocidal antibodies to 2-mercaptoethanol in human cholera. J. infect. Dis. 121, S25-S30.

MILES, A.A. & MISRA, S.S. (1938). The estimation of the bactericidal power of the blood. J. Hyg. 38, 732, Camb.

MILLER, C.E., WONG, K.H., FEELEY, J.C. & FORLINES, M.E. (1972). Immunological conversion of Vibrio cholerae in gnotobiotic mice. Infect. Immun. 6, 739-742.

MOSLEY, W.H. (1969). The role of immunity in cholera: A review of epidemiological and serological studies. Tex. Rep. Biol. Med. 27, suppl. 1, 227-241.

MOSLEY, W.H., BENENSON, A.S. & BARUI, R. (1968,a). A serological survey for cholera antibodies in rural East Pakistan. 2. A comparison of antibody titres in the immunised and control populations of a cholera-vaccine field trial area and the relation of antibody titre to cholera case rate. Bull. Wld Hlth Org. 38, 327-334.

MOSLEY, W.H., AHMAD, S., BENENSON, A.S. & AHMED, A. (1968,b). The relationship of vibriocidal antibody titre to susceptibility to cholera in family contacts of cholera patients. Bull. Wld Hlth Org. 38, 777-785.

MOSLEY, W.H. & AHMED, A. (1969). Active and passive immunisation in the adult rabbit ileal loop model, as an assay for production of antitoxin immunity by cholera vaccines. J. Bact. 100, 547-549.

MOSLEY, W.H., McCORMACK, W.M., FAHIMUDDIN, M., AZIZ, K.M.A., RAHMAN, A.S.M.M., CHOWDHURY, A.K.M.A., MARTIN, A.R., FEELEY, J.C. & PHILLIPS, R.A. (1969,a). Report on the 1966-67 cholera vaccine field trial in rural East Pakistan. 1. Study design and results of the first year of observation. Bull. Wld Hlth Org. 40, 177-185.

MOSLEY, W.H., McCORMACK, W.M., AHMED, A., CHOWDHURY, A.K.M.A. & BARUI, R.K. (1969,b). Report of the 1966-67 cholera vaccine field trial in rural East Pakistan. 2. Results of the serological surveys in the study population - the relationship of case rate to antibody titre and an estimate of the inapparent infection rate with Vibrio cholerae. Bull. Wld Hlth Org. 40, 187-197.

MOSLEY, W.H., AZIZ, K.M.S. & AHMED, A. (1970,a). Serological evidence for the identity of the vascular permeability factor and ileal loop toxin of Vibrio cholerae. J. infect. Dis. 121, 243-250.

MOSLEY, W.H., WOODWARD, W.E., AZIZ, K.M.A., RAHMAN, A.S.M.M., CHOWDHURY, A.K.M.A., AHMED, A. & FEELEY, J.C. (1970,b). The 1968-69 cholera vaccine field trial in rural East Pakistan. Effectiveness of monovalent Ogawa and Inaba vaccines and a purified Inaba antigen, with comparative results of serological and animal protection tests. J. infect. Dis. 121, S1-S9.

MOSLEY, W.H., AZIZ, K.M.A., RAHMAN, A.S.M.M., CHOWDHURY, A.K.M.A., AHMED, A. & FAHIMUDDIN, M. (1972). Report of the 1966-67 cholera vaccine trial in rural East Pakistan. 4. Five years of observation with a practical assessment of the role of cholera vaccine in cholera control programmes. Bull. Wld Hlth Org. 47, 229-238.

MOSLEY, W.H., AZIZ, K.M.A., RAHMAN, A.S.M.M., CHOWDHURY, A.K.M.A. & AHMED, A. (1973). Field trials of monovalent Ogawa and Inaba cholera vaccines in rural Bangladesh - 3 years observation. Bull. Wld Hlth Org. 49, 381-387.

MOSS, J., MANGANIELLO, V.C. & FISHMAN, P.H. (1977). Enzymatic and chemical oxidation of gangliosides in cultured cells: Effects of choleragen. Biochemistry, 16, 1876-1881.

MOSS, J. & VAUGHAN, M. (1977). Mechanism of action of choleragen: Evidence for ADP-ribosyltransferase activity with arginine as an acceptor. J. biol. Chem. 252, 2455-2457.

MUKERJEE, S. (1963). The bacteriophage susceptibility test in differentiating Vibrio cholerae and Vibrio eltor. Bull. Wld Hlth Org. 28, 333-336.

NAGY, L.K. (1972). The effect of deoxycholate on cholera vaccine. Prog. Immunobiol. Stand. 5, 341-347.

NAKASHIMA, Y., NAPIORKOWSKI, P., SCHOFER, D.E. & KONIGSBERG, W.H. (1976). Primary structure of the B subunit of cholera enterotoxin. F.E.B.S. Letters, 68, 275-278.

NALIN, D.R. (1975). Recent advances in cholera pathophysiology and therapeutics. Prog. Drug Res. 19, 563-569.

NELSON, E.T., CLEMENTS, J.D. & FINKELSTEIN, R.A. (1976). Vibrio cholerae adherence and colonisation in experimental cholera: Electron microscope studies. Infect. Immun. 14, 527-547.

NEOH, S.H. & ROWLEY, D. (1970). The antigens of Vibrio cholerae involved in the vibriocidal action of antibody and complement. J. infect. Dis. 121, 505-513.

NEOH, S.H. & ROWLEY, D. (1972). Protection of infant mice against cholera by antibodies to three antigens of Vibrio cholerae. J. infect. Dis. 126, 41-47.

NORRIS, H.T. & MAJNO, G. (1968). On the role of the ileal epithelium in the pathogenesis of experimental cholera. Am. J. Pathol. 53, 263-275.

NORTHRUP, R.S., BIENENSTOCK, J. & TOMASI, T.B. (1970). Immunoglobulins and antibody activity in the intestine and serum in cholera. I. Analysis of immunoglobulins in cholera stool. J. infect. Dis. 121, S137-S141.

NORTHRUP, R.S. & CHISARI, F.V. (1972). Response of monkeys to immunisation with cholera toxoid and vaccine: reversion of cholera toxoid. J. infect. Dis. 125, 471-479.

NORTHRUP, R.S. & FAUCI, A.S. (1972). Adjuvant effect of cholera enterotoxin on the immune response of the mouse to sheep red blood cells. J. infect. Dis. 125, 672-673.

OGONUKI, H., HASHIZUME, S. & TAKASHASHI, B. (1967). Preparation and laboratory tests of oil-adjuvant cholera vaccine. Bull. Wld Hlth Org. 37, 729-736.

OHTOMO, N., MURAOKA, T., TASHIRO, A., ZINNAKA, Y. & AMAKO, K. (1976). Size and structure of the cholera toxin molecule and its subunits. J. infect. Dis. 133, S31-S41.

OSEASOHN, R., AHMED, S., ISLAM, M.A. & RAHMAN, A.S.M.M. (1966). Clinical and bacteriological findings among families of cholera patients. Lancet, i, 340-342.

PANT, K.D. (1968). Cell wall antigens of Vibrio cholerae. Isolation, properties and fractionation of protein-LPS complex. Indian J. Micro. 8, 1-10.

PANT, K.D. & SHRIVASTAVA, D.L. (1960). Studies in immunochemistry of V. cholerae. II. Preparation of polysaccharides from growth on agar. Indian J. med. Res., 48, 677-682.

PARKINSON, D.K., EBEL, H., DiBONA, D.R. & SHARP, G.W.G. (1972). Localisation of the action of cholera toxin on adenyl cyclase in mucosal epithelial cells of rabbit intestine. J. clin. Invest. 51, 2292-2298.

PEARSON, C.M. & WOOD, F.D. (1959). Studies of polyarthritis and other lesions induced in rats by injection of mycobacterial adjuvant. I. General clinical and pathologic characteristics and some modifying factors. Arthr. & Rheum. 2, 440-459.

PERKINS, F.T., SHEFFIELD, F.W., OUTSCHOORN, A.S. & HEMSLEY, D.A. (1973). An international collaborative study on the measurement of the opacity of bacterial suspensions. J. biol. Stand. 1, 1-10.

PETERSON, J.W., LoSPALLUTO, J.J. & FINKELSTEIN, R.A. (1972). Localisation of cholera toxin in vivo. J. infect. Dis. 126, 617-628.

PHILIPPINES CHOLERA COMMITTEE (1968). A controlled field trial of the effectiveness of various doses of cholera El Tor vaccine in the Philippines. Bull. Wld Hlth Org. 38, 917-923.

PHILIPPINES CHOLERA COMMITTEE (1973,a). A controlled field trial of the effectiveness of monovalent classical and El Tor cholera vaccines in the Philippines. Bull. Wld Hlth Org. 49, 13-19.

PHILIPPINES CHOLERA COMMITTEE (1973,b). A controlled field trial on the effectiveness of the intradermal and subcutaneous administration of cholera vaccine in the Philippines. Bull. Wld Hlth Org. 49, 389-394.

PIERCE, N.F. (1976). Intestinal immunisation with soluble bacterial antigens: The example of cholera toxoid. Ciba Foundation Symp. 42, 129-147.

PIERCE, N.F., BANWELL, J.G., SACK, R.B., MITRA, R.C. & MONDAL, A. (1970). Magnitude and duration of antitoxic response to human infection with Vibrio cholerae. J. infect. Dis. 121, S31-S35.

PIERCE, N.F., GREENOUGH, W.B. & CARPENTER, C.C.J. (1971). Vibrio cholerae enterotoxin and its mode of action. Bact. Revs. 35, 1-13.

PIERCE, N.F., KANIECKI, E.A. & NORTHRUP, R.S. (1972). Protection against experimental cholera by antitoxin. J. infect. Dis. 126, 606-616.

PIERCE, N.F. & MONDAL, A. (1974). Clinical features of cholera. In Cholera, chapter 10, ed. Barua, D. & Burrows, W., W.B. Saunders Company.

PIERCE, N.F. & REYNOLDS, H.Y. (1974). Immunity to experimental cholera. 1. Protective effect of humoral IgG antitoxin demonstrated by passive immunisation. J. Immun. 113, 1017-1023.

PIERCE, N.F. & GOWANS, J.L. (1975). Cellular kinetics of the intestinal immune response to cholera toxoid in rats. J. exp. Med. 142, 1550-1563.

PIERCE, N.F. & REYNOLDS, H.Y. (1975). Immunity to experimental cholera. II. Secretory humoral antitoxin response to local and systemic toxoid administration. J. infect. Dis. 131, 383-389.

PIERCE, N.F. & SACK, R.B. (1977). Immune response of the intestinal mucosa to cholera toxoid. J. infect. Dis. 136, S113-S117.

PIERCE, N.F., SACK, R.B. & SIRCAR, B.K. (1977). Immunity to experimental cholera. III. Enhanced duration of protection after sequential parenteral-oral administration of toxoid to dogs. J. infect. Dis. 135, 888-896.

PITKIN, D. & ACTOR, P. (1972). Immunity to Vibrio cholerae in the mouse. I. Passive protection of newborn mice. Infect. Immun. 5, 428-432.

PITTMAN, M. & FEELEY, J.C. (1963). Protective activity of cholera vaccines against El Tor cholera vibrios. Bull. Wld Hlth Org. 28, 379-383.

POLLITZER, R. (1959). Cholera. Wld Hlth Org., Monograph series, No. 43, Geneva.

RAPPAPORT, R.S., BOND, G. McCANN, T., RUBIN, B.A. & TINT, H. (1974). Development of a purified cholera toxoid. II. Preparation of a stable, antigenic toxoid by reaction of purified toxin with glutaraldehyde. Infect. Immun. 9, 304-317.

RAPPAPORT, R.S., PIERZCHALA, W.A., BONDE, G., McCANN, T. & RUBIN, B.A. (1976). Development of a purified cholera toxoid. III. Refinements in purification of toxin and methods for the determination of residual somatic antigen. Infect. Immun. 14, 687-693.

RAZIUDDIN, S. & KAWASAK, T. (1976). Biochemical studies on the cell wall lipopolysaccharides (O-antigens) of Vibrio cholerae 569B (Inaba) and El Tor (Inaba). Biochim. biophys. Acta. 431, 116-126.

REDMOND, J.W. (1975). 4-amino-4,6-dideoxy-D-mannose (D-perosamine): A component of the lipopolysaccharide of Vibrio cholerae 569B (Inaba). F.E.B.S. Letters, 50, 147-149.

RICHARDSON, S.H. (1969). Factors influencing in vitro skin permeability factor production by Vibrio cholerae. J. Bact. 100, 27-34.

RICHARDSON, S.H. & EVANS, D.J. (1968). Isolation of cholera toxins by dextran sulphate precipitation. J. Bact. 96, 1443-1445.

RICHARDSON, S.H., EVANS, D.G. & FEELEY, J.C. (1970). Biochemistry of Vibrio cholerae virulence. I. Purification and biological properties of PF/cholera enterotoxin. Infect. Immun. 1, 546-554.

RICHARDSON, S.H. & NOFTLE, K.A. (1970). Purification and properties of permeability factor/cholera enterotoxin from complex and synthetic media. J. infect. Dis. 121, S73-S79.

RODA, L.G., TOMASI, M., BATTISTINI, A., LUZZI, I., MASTRANTONIO, P., ZAMPIERI, A. & D'AGNOLO, G. (1977). Heterogenicity of purified cholera toxin. Biochim. biophys. Acta. 492, 303-315.

SACK, R.B., BARUA, D., SAXENA, R. & CARPENTER, C.C.J. (1966). Vibriocidal and agglutinating antibody patterns in cholera patients. J. infect. Dis. 116, 630-640.

SACK, R.B. & MILLER, C.E. (1969). Progressive changes of vibrio serotypes in germ-free mice infected with Vibrio cholerae. J. Bact. 99, 688-695.

SALETTI, M. & RICCI, A. (1974,a). Experiments with cholera toxin detoxified with glutaraldehyde. Bull. Wld Hlth Org. 51, 633-639.

SALETTI, M. & RICCI, A. (1974,b). Experiments on monkeys with cholera toxin partially purified and detoxified with formal and glycine. Bull. Wld Hlth Org. 53, 641-645.

SANYAL, S.C. & MUKERJEE, S. (1969). Live oral cholera vaccine: report of a trial on human volunteer subjects. Bull. Wld Hlth Org. 40, 503-511.

SASAKI, S., OHNISHI, N., SUZUKI, R., ADACHI, K., MIYASHITA, M., SHIMAMURA, T., TAZUME, S., MAEDA, R. & TAKAHASHI, T. (1970). The relation between the persistence of El Tor bivrio in the intestines of germ-free mice and so-called copro-antibody. J. infect. Dis. 121, S124-S131.

SATTLER, J., WIEGANDT, H., STAERK, J., KRANZ, T., RONNEBERGER, H.J., SCHMIDTBERGER, R. & ZILG, H. (1975). Studies on the subunit structure of choleragen. Eur. J. Biochem. 57, 309-316.

SCHRANK, G.D. & VERWEY, W.F. (1976). Distribution of cholera organisms in experimental Vibrio cholerae infections: proposed mechanisms of pathogenesis and antibacterial immunity. Infect. Immun. 13, 195-203.

SCHWARTZ, C.J., KIMBERG, D.V. & WARE, P. (1975). Adenylate cyclase in intestinal crypt and villus cells: stimulation by cholera enterotoxin and prostaglandin E_1. Gastroenterology, 68, 94-104.

SEDLACEK, H.H., STÄRK, J., SEILER, F.R., ZEIGLER, W. & WIEGANDT, H. (1976). Cholera toxin induced redistribution of sialoglycolipid receptor at the lymphocyte membrane. F.E.B.S. Letters 61, 272-276.

SEVER, J.L. (1962). Application of a microtechnique to viral serological investigations. J. Immunol. 88, 320-329.

SHARP, G.W.G. & HYNIE, S. (1971). Stimulation of intestinal adenyl cyclase by cholera toxin. Nature, 229, 266-269, Lond.

SHRIVASTAVA, D.L. (1965). Immunochemistry of vibrio antigens. In Proc. Cholera Res. Symp., Pub. Hlth Serv. Publication No. 1328. pp.244-247. U.S. Government Printing Office, Washington, D.C.

SINHA, V.D. & BHASKARON, K. (1973). Immunity in experimental cholera: Effect of parenteral immunisation with vaccines and toxoid. Bull. Wld Hlth Org. 49, 605-613.

SOLOMON, G.E. & TAUB, R.N. (1976). Enteric migration of radiolabeled lymphocytes. Cell. Immun. 23, 108-116.

SPYRIDES, G.J. & FEELEY, J.C. (1970). Concentration and purification of cholera exotoxin by absorption on aluminium compound gels. J. infect. Dis. 121, S96-S99.

STEEL, E.J., CHAICUMPA, W. & ROWLEY, D. (1974). Isolation and biological properties of three classes of rabbit antibody to Vibrio cholerae. J. infect. Dis. 130, 93-103.

STEWART-TULL, D.E.S., SHIMONO, T., KOTANI, S., KATO, M., OGAWA, Y., YAMAMURA, Y., KOGA, T. & PEARSON, C.M. (1975). The adjuvant activity of a non-toxic, water-soluble glycopeptide present in large quantities in the culture filtrate of Mycobacterium tuberculosis strain DT. Immunology, 29, 1-15.

STEWART-TULL, D.E.S., SHIMONO, T., KOTANI, S. & KNIGHTS, B.A. (1976). Immunosuppressive effect in Mycobacterial adjuvant emulsions of mineral oils containing low molecular weight hydrocarbons. Int. Archs. Allergy appl. Immun. 52, 118-128.

SUR, P., MAITI, M. & CHATTERJEE, S.N. (1974). Physicochemical studies of Vibrio cholerae cell envelope fraction. Indian J. exp. Biol. 12, 479-483.

SVENNERHOLM, A.M. & HOLMGREN, J. (1976). Synergistic protective effect in rabbits of immunisation with Vibrio cholerae lipopolysaccharide and toxin/toxoid. Infect. Immun. 13, 735-740.

SVENNERHOLM, A.M. & HOLMGREN, J. (1977). Immunoglobulin and specific antibody synthesis in vitro by enteral and non-enteral lymphoid tissues after subcutaneous cholera immunisation. Infect. Immun. 15, 360-369.

SVENNERHOLM, A.M., HOLMGREN, J., HANSON, L.Å., LINDBLAD, B.S., QUERESHI, F. & RAHIMTOOLA, R.J. (1977). Boosting secretory IgA antibody responses in man by parenteral cholera vaccination. Scand. J. Immunol. 6, 1346-1349.

TAYLOR, J.O., KINZIE, J., HARE, R. & HARE, K. (1968). Measurement of sodium fluxes in human small intestine. Fedn. Proc. 27, 386 (Abstr. No. 0964).

THOMSON, R.O., WALKER, P.D. & KNIGHT, P.A. (1972). The production of a vaccine based on Craig's toxin. Prog. immunobiol. Stand. 5, 335-340.

UJIIYE, A., NAKATOMI, M., UTSUNOMIYA, A., MITSUI, K., SOGAME, S., IWANAGA, M. & KOBARI, K. (1968). Experimental cholera in mice. I. First report on the oral infection. Trop. Med. 10, 65-71.

UNGAR, J., STANIC, M., CHARIATTE, N. & VARALLYAY, S.J. (1970). Studies on cholera toxin and antitoxin. J. gen. Micro. 64, 1-10.

VAN HEYNINGEN, S. (1974,b). Cholera toxin. i. Interaction of subunits with ganglioside Gm_1. Science 183, 656-657.

VAN HEYNINGEN, S. (1976). The subunits of cholera toxin: structure, stochiometry and function. J. infect. Dis. 133, S5-S13.

VAN HEYNINGEN, S. & KING, C.A. (1975). Subunit A from cholera toxin is an activator of adenylate cyclase in pigeon erythrocytes. Biochem. J. 146, 269-271.

VAN HEYNINGEN, W.E. (1974,a). Gangliosides as membrane receptors for tetanus toxin, cholera toxin and serotonin. Nature, 249, No. 5456, 415-417, Lond.

VAN HEYNINGEN, W.E., CARPENTER, C.C.J., PIERCE, N.F. & GREENOUGH, W.B. (1971). Deactivation of cholera toxin by ganglioside. J. infect. Dis. 124, 415-418.

VAN HEYNINGEN, W.E., VAN HEYNINGEN, S. & KING, C.A. (1976). The nature and action of cholera toxin. Ciba Foundation Symp. 42, 73-88.

VAUGHAN-WILLIAMS, E.M. & DOHADWALLA, A.N. (1969). The appearance of a choleragenic agent in the blood of infant rabbits infected intestinally with Vibrio cholerae, demonstrated by cross-circulation. J. infect. Dis. 120, 658-663.

VERWEY, W.F., WATANABE, Y., PHILLIPS, P.E. & WILLIAMS, H.R. (1965). The partial purification and some of the properties of the mouse protective antigen of Inaba subtype of Vibrio cholerae (El Tor). In Proc. Cholera, Res. Symp., Pub. Hlth Serv. Publication No. 1328, pp.259-263. U.S. Government Printing Office, Washington, D.C.

VERWEY, W.F., WATANABE, Y., GUCKIAN, J.C., WILLIAMS, H.R., PHILLIPS, P.E., ROCHA, S.S. & BRIDGEFORTH, E.B. (1969). Serological responses of human volunteers to cholera vaccine. Tex. Rep. Biol. Med. 27, suppl. 1, 243-274.

VERWEY, W.F., CRAIG, J.P., FEELEY, J.C., GREENOUGH, W.B. GUCKIAN, J.C. & PIERCE, N.F. (1972). Response of human volunteers to the parenteral injection of partially purified cholera toxoid: clinical and serological observations. In Symp. on cholera, Proc. 7th Joint Conference, U.S.-Japan Cooperative Med. Sci. Prog. pp.99-100. Nat. Inst. Hlth. Bethesda, M.D.

VERWEY, W.F., GUCKIAN, J.C., CRAIG, J., PIERCE, N., PETERSON, J. & WILLIAMS, H. (1975). The current status of cholera toxoid research in the United States. Prog. Drug Res. 19, 602-611.

VOLKHEIMER, C. & SCHULZ, F.H. (1968). The phenomena of persorption. Digestion, 1, 213-218.

WALDMAN, R.H., BENCIC, Z., SAKAZAKI, R., SINHA, R., GANGULY, R., DEB, B.C. & MUKERJEE, S. (1971). Cholera immunology. I. Immunoglobulin levels in serum, fluid from the small intestine and faeces from patients with cholera and noncholeraic diarrhoea during illness and convalescence. J. infect. Dis. 123, 579-586.

WALDMAN, R.H., BENCIC, Z., SINHA, R., DEB, B.C., SAKAZAKI, R., TAMURA, K., MUKERJEE, S. & GANGULY, R. (1972). Cholera immunology. II. Serum and intestinal secretion antibody response after naturally occurring cholera. J. infect. Dis. 126, 401-407.

WATANABE, Y. & VERWEY, W.F. (1965). The preparation and properties of a purified mouse-protective lipopolysaccharide from the Ogawa subtype of the El Tor variety of Vibrio cholerae. In Proc. Cholera Res. Symp., Pub. Hlth. Serv. Publication No. 1328, pp.253-259, U.S. Government Printing Office, Washington, D.C.

WATANABE, Y., VERWEY, W.F., GUCKIAN, J.C., WILLIAMS, H.R., PHILLIPS, P.E. & ROCHA, S.S. (1969). Some of the properties of mouse protective antigens derived from Vibrio cholerae. Tex. Rep. Biol. Med. 27, suppl. 1, 275-298.

WAKSMAN, B.H., PEARSON, C.M. & SHARP, J.T. (1960). Studies of arthritis and other lesions induced in rats by injection of mycobacterial adjuvants. II. Evidence that the disease is a disseminated immunologic response to exogenous antigen. J. Immunol. 85, 403-417.

WEISER, M.M. & QUILL, H. (1975). Intestinal villus and crypt cell responses to cholera toxin. Gastroenterology, 69, 479-482.

WESTPHAL, O. (1975). Bacterial endotoxins. Int. Archs. Allergy appl. Immun. 49, 1-43.

WOODWARD, W.E. (1971). Cholera reinfection in man. J. infect. Dis. 123, 61-66.

WU, A., SICARD, R.E., APRILLE, J.R. & WALKER, W.A. (1976). The immunologic control mechanism against cholera toxin. II. Stimulation of adenylate cyclase without fluid secretion. Biochem. biophys. Res. Comm. 72, 1414-1422.

YANG, G.C.H., SHRANK, G.D. & FREEMAN, B.A. (1977). Purification of flagellar cores of Vibrio cholerae. J. Bact. 129, 1121-1128.

YOSHIOKA, M., TAKAHASH, K., HIRANO, T., KAZUNC, Y. TAKEUCHI, S., AKIBA, Y., GHODA, A., SUZUKI, T. & SUZUKI, Y. (1970). Separation of protective bacterial substances against experimental vibrio infection. J. infect. Dis. 121, S45-S49.

APPENDICES

Appendix 1

Ileal loop response to injection of graded doses of N.I.H. toxin

N.I.H. toxin dose (μg)	ml fluid/ cm ileal loop	$\bar{x}$	SEM
5	0.37 0.65	0.51	0.14
10	0.35 0.6	0.48	0.12
12.5	0.54 0.49	0.515	0.02
25	0.52 0.51	0.515	0
50	0.39 0.43 0.52 0.54	0.47	0.04
100	0.52 0.56	0.54	0.02
200	0.48 0.51	0.495	0.01
250	0.75 0.73 0.66 0.59 0.74	0.69	0.03
500	0.7 0.65 0.58 0.71 0.7	0.67	0.02
1000	0.79 0.71 0.64 0.6 0.69 0.79	0.7	0.03
0		0.37	0.01

APPENDIX 2

Table 1. Fluid accumulation in ileal loops on challenge with NIH toxin

Guinea-pig Code No. DST GP	Vaccine	Route of administration	Time months	mg toxin:- 1.0 ml/cm	1.0 $\bar{x}$ s	0.5 ml/cm	0.5 $\bar{x}$ s	0.25 ml/cm	0.25 $\bar{x}$ s	0 ml/cm	0 $\bar{x}$ s
321	60 µg Toxoid in IA	oral x 1		0.75	0.62	0.72	0.6	0.70	0.61	0.43	0.47
322		0.3 ml	1	0.68		0.68		0.66		0.54	
323				0.50	0.113	0.54	0.121	0.50	0.089	0.58	0.104
324				0.56		0.46		0.57		0.35	
325	60 µg Toxoid in	oral x 2		0.43		0.40		0.34		0.24	
326	IA + booster after	0.3 ml	2	0.54	0.5	0.54	0.5	0.52	0.44	0.38	0.29
327	6 weeks			0.46		0.48		0.44		0.15	
328				0.57	0.065	0.58	0.078	0.47	0.075	0.38	0.113
329	60 µg Toxoid in IA	oral x 3		0.5		0.47		0.51		0.43	
330	+ boosters after 6	0.3 ml	3	0.75	0.62	0.77	0.63	0.69	0.63	0.06	0.26
331	& 10 weeks			0.45		0.59		0.56		0.07	
332				0.79	0.172	0.68	0.128	0.74	0.108	0.47	0.223
344	60 µg Toxoid with	oral x 1		0.65		0.74		0.75		0.64	
345	300 µg ST208 in IA	0.3 ml	1	0.60	0.66	0.60	0.66	0.60	0.68	0.46	0.55
346				0.75		0.68		0.75		0.61	
347				0.64	0.063	0.63	0.061	0.61	0.084	0.51	0.084
348	60 µg Toxoid with	oral x 2		0.25		0.15		0.19		0.06	
349	300 µg ST208 in IA	0.3 ml	2	0.54	0.42	0.50	0.4	0.57	0.42	0.31	0.12
350	+ booster after 6			0.38		0.39		0.37		0.12	
351	weeks, 60 µg Toxoid in IA			0.51	0.133	0.54	0.175	0.55	0.177	0	0.134
352	60 µg Toxoid with	oral x 3		0.22		0.21		0.30		0	
353	300 µg ST208 in IA	0.3 ml		0.74	0.51	0.79	0.54	0.08	0.39	0.36	0.22
354	+ boosters after 6			0.52		0.59		0.56		0.44	
355	& 10 weeks, 60 µg Toxoid in IA			0.54	0.214	0.55	0.241	0.63	0.178	0.1	0.208

Table 2. Fluid accumulation in ileal loops on challenge with NIH toxin

Guinea-pig Code No. DST GP	Vaccine	Route of administration	Time months	mg toxin:- 1.0 ml/cm	1.0 $\bar{x}$ s	0.5 ml/cm	0.5 $\bar{x}$ s	0.25 ml/cm	0.25 $\bar{x}$ s	0 ml/cm	0 $\bar{x}$ s
365	100 µg Toxoid in IA	i/m x 1		0.64		0.65		0.65		0.58	
366		0.5 ml	1	0.36	0.55	0.77	0.66	0.79	0.63	0.71	0.63
367				0.66	0.137	0.68	0.095	0.55	0.119	0.63	0.059
368				0.54		0.54		0.53		0.59	
369	100 µg Toxoid in IA	i/m x 1		0.36		0.42		0.35		0.13	
370	+ booster after 6	0.5 ml;	2	0.44	0.40	0.49	0.44	0.42	0.40	0.31	0.22
371	weeks, 60 µg Toxoid	oral x 2		0.38	0.041	0.49	0.031	0.40	0.036	0.15	0.091
372	in IA	0.3 ml		0.44		0.44		0.43		0.28	
373	100 µg Toxoid in IA	i/m x 1		0.70		0.73		0.73		0.53	
374	+ boosters after 6 &	0.5 ml;	3	0.71	0.68	0.69	0.69	0.69	0.65	0.36	0.42
375	10 weeks, 60 µg	oral x 2		0.60	0.056	0.59	0.071	0.47	0.119	0.34	0.086
376	Toxoid in IA	0.3 ml		0.72		0.75		0.70		0.44	
386	100 µg Toxoid with	i/m x 1		0.19		0.19		0.28		0.21	
387	500 µg ST208 in IA	0.5 ml	1	0.55	0.42	0.59	0.44	0.58	0.44	0.44	0.41
388				0.44	0.161	0.42	0.180	0.39	0.136	0.49	0.136
389				0.51		0.55		0.53		0.50	
390	100 µg Toxoid with	i/m x 1		0.50		0.43		0.48		0.34	
391	500 µg ST208 in IA +	0.5 ml;	2	0.36	0.38	0.36	0.36	0.16	0.30	0.24	0.24
392	booster after 6	oral x 1		0.18	0.145	0.18	0.128	0.15	0.168	0.13	0.086
393	weeks, 60 µg Toxoid in IA	0.3 ml		0.47		0.47		0.40		0.24	
394	100 µg Toxoid with	i/m x 1		0.56		0.60		0.08		0.45	
395	500 µg ST208 in IA	0.5 ml;	3	0.17	0.41	0.38	0.48	0.31	0.33	0.34	0.37
396	+ boosters after 6	oral x 2		0.50	0.171	0.56	0.117	0.50	0.188	0.42	0.077
397	& 10 weeks, 60 µg Toxoid	0.3 ml		0.43		0.38		0.45		0.28	
Controls											
628	Non-immunized			0.79		0.8		0.75		0.51	
638				0.71	0.70	0.69	0.69	0.69	0.60	0.50	0.508
639				0.64	0.077	0.65	0.072	0.66	0.207	0.44	0.047
640				0.60		0.58		0.59		0.56	
641				0.69		0.71		0.20		0.48	
642				0.79		0.70		0.74		0.56	

APPENDIX 3

Table 1. Fluid accumulation in ileal loops challenged with 2×10^6 V. cholerae

Guinea-pig Code no. DST GP	Vaccine	Route of administration	Time months	Challenge loop ml/cm	Challenge loop $\bar{x}$ s	Control loop ml/cm	Control loop $\bar{x}$ s
410-413	60 µg Toxoid in IA	oral x 1		0.83		0.19	
		0.3 ml	1	0.8	0.85	0.68	0.49
				0.81	0.074	0.31	0.28
				0.96		0.77	
414-417	60 µg Toxoid in IA +	oral x 2		0.71	0.76	0.63	0.57
	booster after 6	0.3 ml	2	0.74	0.04	0.55	0.05
	weeks			0.8		0.6	
				0.78		0.51	
418-421	60 µg Toxoid in IA +	oral x 3		0.69	0.85	0.31	0.6
	boosters after 6 &	0.3 ml	3	0.98	0.15	0.77	0.25
	10 weeks			0.89		0.73	
434-437	60 µg Toxoid with	oral x 1		0.74	0.76	0.63	0.65
	300 µg ST208 in IA	0.3 ml	1	0.78	0.02	0.68	0.02
				0.75		0.65	
438-441	60 µg Toxoid with	oral x 2		0.68	0.73	0.78	0.72
	300 µg ST208 in IA +	0.3 ml	2	0.91	0.15	0.75	0.07
	booster after 6 weeks			0.62		0.64	
442-445	60 µg Toxoid with	oral x 3		0.75	0.63	0.34	0.57
	300 µg ST208 in IA +	0.3 ml	3	0.73	0.23	0.68	0.16
	boosters after 6 &			0.29		0.57	
	10 weeks			0.75		0.7	

IA = incomplete adjuvant: Octadecane, 2.5 ml; Bayol F, 2.5 ml; Arlacel, 1.0 ml; saline, 2.5 ml; toxoid, 1.0 ml.

Table 2. Fluid accumulation in ileal loops challenged with 2 x 10^6 V. cholerae

Guinea-pig Code no. DST GP	Vaccine	Route of administration	Time months	Challenge loop ml/cm	Challenge loop $\bar{x}$	Challenge loop s	Control loop ml/cm	Control loop $\bar{x}$	Control loop s
422-425	100 µg Toxoid in IA	i/m x 1		0.74			0.81		
		0.5 ml	1	0.87	0.81		0.7	0.79	
				0.82	0.07		0.87	0.09	
426-429	100 µg Toxoid in IA	i/m x 1		0.8	0.76		0.66	0.53	
	+ booster after 6	0.5 ml;	2	0.8	0.06		0.6	0.17	
	weeks	oral x 1		0.69			0.33		
		0.3 ml							
430-433	100 µg Toxoid in IA	i/m x 1		0.68	0.74		0.58	0.68	
	+ boosters after 6	0.5 ml;	3	0.74	0.07		0.67	0.11	
	& 10 weeks	oral x 2		0.82			0.8		
		0.3 ml							
446-449	100 µg Toxoid with	i/m x 1		0.83	0.86		0.94	0.8	
	500 µg ST208 in IA	0.5 ml	1	0.82	0.05		0.82	0.14	
				0.92			0.65		
450-453	100 µg Toxoid with	i/m x 1		0.67	0.79		0.14	0.42	
	500 µg ST208 in IA	0.5 ml;	2	0.69	0.13		0.6	0.27	
	+ booster after 6	oral x 1		0.85			0.71		
	weeks	0.3 ml		0.95			0.23		
453-457	100 µg Toxoid with	i/m x 1		0.63	0.69		0.74	0.55	
	500 µg ST208 in IA	0.5 ml;	3	0.74	0.08		0.56	0.1407	
	+ boosters after 6	oral x 1		0.77			0.41		
	& 10 weeks	0.3 ml		0.61			0.49		

IA = incomplete adjuvant: Octadecane, 2.5 ml; Bayol F, 2.5 ml; Arlacel, 1.0 ml; saline, 2.5 ml; Wellcome toxoid, 1.0 ml.

Table 3. Fluid accumulation in ileal loops challenged with 2×10^6 *V. cholerae*

Guinea-pig Code no. DST GP	Vaccine	Route of administration	Time months	Challenge loop ml/cm	Challenge loop $\bar{x}$ s	Control loop ml/cm	Control loop $\bar{x}$ s
458-461	60 µg Toxoid with 6×10^4 *V.cholerae* in IA	oral x 1 0.3 ml	1	0.94 0.88 0.86 0.84	0.88 0.04	0.67 0.71 0.75 0.67	0.7 0.04
462-465	60 µg Toxoid with 6×10^4 *V.cholerae* in IA + booster after 6 weeks	oral x 2 0.3 ml	2	0.89 0.75 0.88 0.68	0.8 0.1	0.76 0.43 0.76 0.68	0.66 0.16
466-469	60 µg Toxoid with 6×10^4 *V.cholerae* in IA + boosters after 6 & 10 weeks	oral x 3 0.3 ml	3	0.39 0.77 0.81 0.77	0.68 0.2	0.58 0.69 0.68 0.22	0.54 0.22
500-503	60 µg Toxoid + 6×10^4 *V.cholerae* with 300 µg ST208 in IA	oral x 1 0.3 ml	1	0.75 0.87 0.85	0.82 0.06	0.67 0.75 0.57	0.66 0.09
504-507	60 µg Toxoid + 6×10^4 *V.cholerae* with 300 µg ST208 in IA + booster after 6 weeks	oral x 2 0.3 ml	2	0.69 0.87 0.86	0.81 0.1	0.52 0.8 0.82	0.71 0.1676
508-511	60 µg Toxoid + 6×10^4 *V.cholerae* with 300 µg ST208 in IA + boosters after 6 & 10 weeks	oral x 3 0.3 ml	3	0.3 0.79 0.86 0.72	0.67 0.25	0.54 0.92 0.7 0.63	0.7 0.16

IA = incomplete adjuvant: Octadecane, 2.5 ml; Bayol F, 2.5 ml; Arlacel, 1.0 ml; 1.9×10^6 *V. cholerae* organisms in saline, 2.5 ml; Wellcome toxoid, 1.0 ml.

Table 4. Fluid accumulation in ileal loops challenged with 2 x 10^6 *V. cholerae*

Guinea-pig Code no. DST GP	Vaccine	Route of administration	Time months	Challenge loop ml/cm	Challenge loop $\bar{x}$ s	Control loop ml/cm	Control loop $\bar{x}$ s
479-482	100 µg Toxoid with 1 x 10^5 *V.cholerae* in IA	i/m x 1 0.5 ml	1	0.9 0.69 0.74 0.68	0.75 0.1	0.81 0.58 0.42 0.62	0.61 0.16
483-486	100 µg Toxoid with 1 x 10^5 *V.cholerae* in IA + booster after 6 weeks	i/m x 1 0.5 ml; oral x 1 0.3 ml	2	0.75 0.69 0.95 0.75	0.78 0.11	0.43 0.58 0.49 0.59	0.52 0.08
487-490	100 µg Toxoid with 1 x 10^5 *V.cholerae* in IA + boosters after 6 & 10 weeks	i/m x 1 0.5 ml; oral x 2 0.3 ml	3	0.73 0.88 0.72 0.81	0.76 0.07	0.57 0.8 0.8 0.81	0.74 0.12
521-524	100 µg Toxoid + 1 x 10^5 *V.cholerae* with 500 µg ST208 in IA	i/m x 1 0.5 ml	1	0.83 0.81 0.87	0.84 0.03	0.64 0.67 0.77	0.69 0.07
525-528	100 µg Toxoid + 1 x 10^5 *V.cholerae* with 500 µg ST208 in IA + booster after 6 weeks	i/m x 1 0.5 ml; oral x 1 0.3 ml	2	0.68 0.9 0.13	0.57 0.4	0.85 0.72 0.71	0.76 0.08
529-532	100 µg Toxoid + 1 x 10^5 *V.cholerae* with 500 µg ST208 in IA + boosters after 6 & 10 weeks	i/m x 1 0.5 ml; oral x 2 0.3 ml	3	0.73 0.83 0.67 0.75	0.74 0.06	0.68 0.45 0.69 0.67	0.62 0.11

IA = incomplete adjuvant: Octadecane, 2.5 ml; Bayol F, 2.5 ml; Arlacel, 1.0 ml; 1.9 x 10^6 *V. cholerae* organisms in saline, 2.5 ml; Wellcome toxoid, 1.0 ml.

Table 5. Fluid accumulation in ileal loops challenged with 2×10^6 *V. cholerae*

Guinea-pig Code no. DST GP	Vaccine	Route of administration	Time months	Challenge loop ml/cm	Challenge loop $\bar{x}$ s	Control loop ml/cm	Control loop $\bar{x}$ s
542-545	6×10^4 *V.cholerae* in IA	oral x 1 0.3 ml	1	0.88 0.9 0.87 1.0	0.91 0.06	0.45 0.77 0.57 0.61	0.6 0.13
546-549	6×10^4 *V.cholerae* in IA + booster after 6 weeks	oral x 2 0.3 ml	2	0.71 0.89 0.8 0.78	0.79 0.07	0.48 0.5 0.12 0.56	0.41 0.2
550-553	6×10^4 *V.cholerae* in IA + boosters after 6 & 10 weeks	oral x 3 0.3 ml	3	1.03 0.68 0.82 0.85	0.84 0.14	0.62 0.43 0.42 0.57	0.51 0.1
584-587	6×10^4 *V.cholerae* with 300 µg ST208 in IA	oral x 1 0.3 ml	1	0.74 1.02 0.82 0.81	0.85 0.12	0.71 0.93 0.7 0.33	0.67 0.25
588-591	6×10^4 *V.cholerae* with 300 µg ST208 in IA + booster after 6 weeks	oral x 2 0.3 ml	2	0.71 0.71 0.8 0.89	0.78 0.09	0.55 0.65 0.57 0.69	0.61 0.06
592-595	6×10^4 *V.cholerae* with 300 µg ST208 in IA + boosters after 6 & 10 weeks	oral x 3 0.3 ml	3	0.75 0.83 0.6 0.71	0.72 0.09	0.67 0.67 0.14 0.69	0.54 0.27

IA = incomplete adjuvant: Octadecane, 2.5 ml; Bayol F, 2.5 ml; Arlacel, 1.0 ml; 1.9×10^6 *V. cholerae* in saline, 3.5 ml.

Table 6. Fluid accumulation in ileal loops challenged with 2×10^6 *V. cholerae*

Guinea-pig Code No. DST GP	Vaccine	Route of administration	Time months	Challenge loop ml/cm	Challenge loop $\bar{x}$ s	Control loop ml/cm	Control loop $\bar{x}$ s
563-566	1×10^5 *V.cholerae* in IA	i/m x 1 0.5 ml	1	0.76 0.95 0.72	0.81 0.12	0.6 0.75 0.24	0.53 0.26
567-570	1×10^5 *V.cholerae* in IA + booster after 6 weeks	i/m x 1 0.5 ml; oral x 1 0.3 ml	2	0.95 0.94 0.82 0.93	0.91 0.06	0.21 0.63 0.24 0.77	0.46 0.78
571-574	1×10^5 *V.cholerae* in IA + boosters after 6 & 10 weeks	i/m x 1 0.5 ml; oral x 2 0.3 ml	3	0.69 0.86 0.78 0.86	0.8 0.08	0.83 0.64 0.7 0.63	0.7 0.09
605-608	1×10^5 *V.cholerae* with 500 µg ST208 in IA	i/m x 1 0.5 ml	1	0.64 0.72 0.76	0.7 0.06	0.69 0.77 0.73	0.73 0.04
609-612	1×10^5 *V.cholerae* with 500 µg ST208 in IA + booster after 6 weeks	i/m x 1 0.5 ml; oral x 1 0.3 ml	2	0.83 0.84 0.86	0.84 0.01	0.6 0.45 0.65	0.57 0.1
613-616	1×10^5 *V.cholerae* with 500 µg ST208 in IA + boosters after 6 & 10 weeks	i/m x 1 0.5 ml; oral x 2 0.3 ml	3	0.91 1.0 0.89 0.74	0.88 0.1	0.12 0.96 0.58 0.68	0.58 0.35

IA = incomplete adjuvant: Octadecane, 2.5 ml; Bayol F, 2.5 ml; Arlacel, 1.0 ml; 1.9×10^6 *V. cholerae* in saline, 3.5 ml.

Table 7. Fluid accumulation in ileal loops challenged with 2×10^6 V. cholerae

Control Guinea-pig Code No. DST GP	Challenge loop ml/cm	$\bar{x}$ s	Control loop ml/cm	$\bar{x}$ s
634	0.76		0.45	
635	0.9		0.6	
636	0.73	0.88	0.64	0.69
644	0.83	0.13	0.83	0.15
645	0.86		0.65	
646	1.11		0.92	
647	1.0		0.75	

Appendix 4

Statistical Analysis of Ileal Loop Test Results

The $\bar{x}$ and standard deviation (s) values for each group of immunised guinea-pigs and unimmunised guinea-pigs was calculated.

The overall s value for group variation was obtained by summing the s values of immunised animals x 4 (no. of animals per group) and the s value of the unimmunised animals x 6 or 7 (no. of animals per group with toxin or V. cholerae challenge respectively), and dividing by the total number of observations.

A 1-tail t-test was used since it was unreasonable to expect a dose of toxin to produce less fluid accumulation than saline. Also, neutralised toxin, due to immunisation, should produce an equal amount of fluid accumulation to saline.

Two different comparisons were applied to the results:-

A. the mean "ml/cm" in loops injected with toxin or V. cholerae were compared to the loops injected with B.G.S. or proteose peptone respectively

B. the mean "ml/cm" in loop in immunised animals was compared to that in unimmunised guinea-pigs.

For comparison A, the formula used was:-

$$\Delta\bar{x} = s.t.\sqrt{\frac{1}{ns} + \frac{1}{nt}}$$

where $\Delta\bar{x} = \bar{x}_t - \bar{x}_s$

$\bar{x}_t$ = ml/cm given by toxin or V. cholerae challenge

$\bar{x}_s$ = ml/cm given by B.G.S. or proteose peptone

n_t = no of loops challenged with toxin or V. cholerae

n_s = no of loops injected with B.G.S. or proteose peptone.

For comparison B, the formula used was:-

$$\Delta\bar{x} = S.t.\sqrt{\frac{1}{n_{im}} + \frac{1}{n_c}}$$

where $\Delta\bar{x} = \bar{x}_c - \bar{x}_{im}$

$\bar{x}_c$ = ml/cm in control guinea-pig loops

$\bar{x}_{im}$ = ml/cm in immunised guinea-pig loops

n_c = no of control guinea-pigs

n_{im} = no of immunised guinea-pigs.

In both cases:-

S = the overall standard deviation for (i) toxin challenged animals or (ii) V. cholerae challenged animals, where appropriate

t = the value obtained from statistical tables at the 5% and 1% probability levels on substituting the appropriate no. of degrees of freedom, e.g. comparing immunised to unimmunised animals on toxin challenge is a 4 + 6 comparison which has 8 degrees of freedom.

With comparison A:-

the $\Delta\bar{x}$ obtained, indicates by what amount $\bar{x}_t$ must exceed $\bar{x}_s$ for the values to be significantly (t at the 5% level) or highly significantly different (t at the 1% level). Protection from toxin or V. cholerae challenge would be indicated in cases where there was no significant difference in the $\bar{x}$ values.

In Tables 19 and 20, and 23-28, the results of this analysis are given as:-

\+ = no significant difference

$\pm$ = significant difference at the P = 5% level

\- = significant difference at the P = 1% level.

With comparison B:-

the $\Delta \bar{x}$ value obtained indicates by what amount $\bar{x}_c$ must exceed $\bar{x}_{im}$ to be significant or highly significantly different. Protection from challenge would be indicated in cases where the $\bar{x}_{im}$ value was highly significantly lower than the $\bar{x}_c$ value.

In Tables 19 and 20, and 23-28, the results of this analysis are given as:-

\+ = significant difference at the P = 1% level

$\pm$ = significant difference at the P = 5% level

\- = no significant difference.